

TARGETED DIFFERENTIATION OF ES CELL INTO SEROTONERGIC NEURONS

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"The measure of greatness in a scientific idea is the extent to which it stimulates thought and opens up new lines of research. "

-Paul Dirac

(Nobel prize winner, Physics, 1933)

ABSTRACT

Serotonin is a neurotransmitter in the central nervous system (CNS), which has a wide range of functions in human physiology. Serotonergic neurons are located in the raphe nuclei of the brain and project to the spinal cord and almost to all areas of the forebrain. We aimed at directing the differentiation of embryonic stem (ES) cells and induced pluripotent stem (iPS) cells into an enriched population of serotonin producing cells to identify novel genes that are essential for the development and function of serotonergic system. To this purpose we differentiated ES cells into serotonin producing neurons. Using RNA isolated at different time points during the course of ES cell differentiation we identified genes specifically enriched in the serotonergic lineage by Affymetrix gene array. To evaluate candidate genes we reprogrammed mouse and rat embryonic fibroblast to iPS cells and subsequently differentiated them to serotonergic neurons. We selected *Cacna2d1*, coding for an $\alpha 2/\delta$ subunit of voltage dependent calcium channels as a most prominent candidate among these genes. *Cacna2d1* is expressed in skeletal muscle, aorta, and in the central nervous system (CNS) in mammals and may be essential for the release of neurotransmitters. Our own immunohistochemical analysis of the *Cacna2d1* expression pattern in mouse brain coincided with the Allen brain atlas and revealed a partial overlap with *Tph2*-expressing cells confirming the relevance of our *in vitro* data and pointing to a possible importance of this channel for serotonergic function. To analyse this role of the *Cacna2d1* protein we used *Cacna2d1* knockout mice and morpholino-knockdown in zebrafish but we did not see any direct influence on the serotonergic phenotype upon depletion of *Cacna2d1*. However, our immunostaining for *Cacna2d1* in zebrafish revealed a time-dependent pattern during early development. *Cacna2d1* expression starts at day3 at the lateral midline of the trunk; presumably in neuromast cells. Concordantly with their characterization as neuromasts, these *Cacna2d1*-positive cells are migrating towards the tail. This spatial and time-dependent expression was confirmed by RT-PCR using specific *Cacna2d1* primers. Moreover, zebrafish showed disturbed migration behaviours of neuromasts after morpholino-knockdown of *Cacna2d1*. Thus, this study clarified that *Cacna2d1* is essential for zebrafish lateral line development but does not affect the establishment of the serotonergic system.

ZUSAMMENFASSUNG

Serotonin ist ein Neurotransmitter im Zentralnervensystem (ZNS), der eine Vielzahl von Funktionen in der menschlichen Physiologie uebernimmt. Serotonerge Neurone befinden sich in den Raphe-Kernen des Hirnstamms und projizieren zum Rückenmark und zu fast allen Bereichen des Vorderhirns. Das Ziel dieser Arbeit war es, serotonerge Neurone durch die Differenzierung von embryonalen Stamm (ES)-Zellen oder induzierten pluripotenten Stammzellen (iPS-Zellen) zu produzieren, um neue Gene, die bei der Entwicklung und Funktion des serotonergen Systems eine Rolle spielen, zu identifizieren. Zu diesem Zweck wurden als erstes Serotonin-produzierende Neurone aus differenzierenden ES-Zellen hergestellt. Mittels eines Affymetrix-Gen-Arrays mit RNA, die zu verschiedenen Zeitpunkten während der ES-Zelldifferenzierung isoliert wurde, konnten Gene identifiziert werden, die in serotonergen Zellen angereichert sind. Um diese Kandidatengene zu evaluieren, wurden embryonale Fibroblasten von Maus und Ratte zu iPS-Zellen umprogrammiert und anschließend in serotonerge Neurone ausdifferenziert. Als vielversprechendstes Kandidatengen wurde *Cacna2d1* gewählt, das eine α_2/δ - Untereinheit von spannungsabhängigen Kalziumkanälen kodiert. *Cacna2d1* wird im Skelettmuskel, der Aorta und im ZNS von Säugetieren exprimiert und kann bei der Freisetzung von Neurotransmittern wesentlich sein. Unsere Analyse des immunohistochemischen *Cacna2d1* Expressionsmusters im Gehirn der Maus ähnelte dem im Allen-Brain-Atlas beschriebenen und zeigte eine teilweise Überlappung mit *Tph2*-exprimierenden Zellen. Dieses Ergebnis bekräftigt die Relevanz unserer *in vitro* Daten, die auf eine mögliche Bedeutung von *Cacna2d1* bei serotonergen Funktionen hinweisen. Als nächstes wurde die Rolle des *Cacna2d1* Proteins diesbezüglich untersucht unter Verwendung von *Cacna2d1* Knockout-Mäusen und Morpholino-Knockdown Zebrafischen. Jedoch konnte kein direkter Einfluss auf einen serotonergen Phänotyp nachgewiesen werden. Die Immunfärbung für *Cacna2d1* im Zebrafisch ergab allerdings ein zeitabhängiges Muster in der Frühentwicklung. *Cacna2d1* zeigt sich am dritten Tag an der seitlichen Mittellinie des Rumpfes; hier vermutlich in Neuromastzellen. Übereinstimmend mit der Charakterisierung als Neuromastzellen migrieren diese *Cacna2d1*-positiven Zellen dann zum Schwanz hin. Diese räumliche und zeitliche Expression konnte mittels RT-PCR unter Verwendung spezifischer Primer für *Cacna2d1* bestätigt werden. Darüber hinaus zeigte der Morpholino-Knockdown Zebrafisch ein gestörtes Migrationsverhalten der Neuromastzellen. Zusammenfassend zeigt die vorliegende Studie,

dass Cacna2d1 bei die Entwicklung des Zebrafisch-Seitenliniensystems eine wichtige Rolle spielt, jedoch nicht bei der Etablierung des serotonergen Systems.

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*“Tell me and I forget, teach me and I may
remember, involve me and I learn.”*

- Benjamin Franklin

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1.INTRODUCTION

1.1) Embryonic stem (ES) cells

ES cells are pluripotent stem cells. They are harvested from the inner cell mass of the pre-implantation blastocyst and have been derived from rodents, primates and humans. Murine ES cells remain undifferentiated when grown in the presence of Leukemia inhibitory factor (LIF) (Smith et al., 1998). When LIF or feeder cells are withdrawn, most types of ES cells differentiate spontaneously to form aggregates which are called embryoid bodies (EB) and are similar to post implantation embryonic tissue.

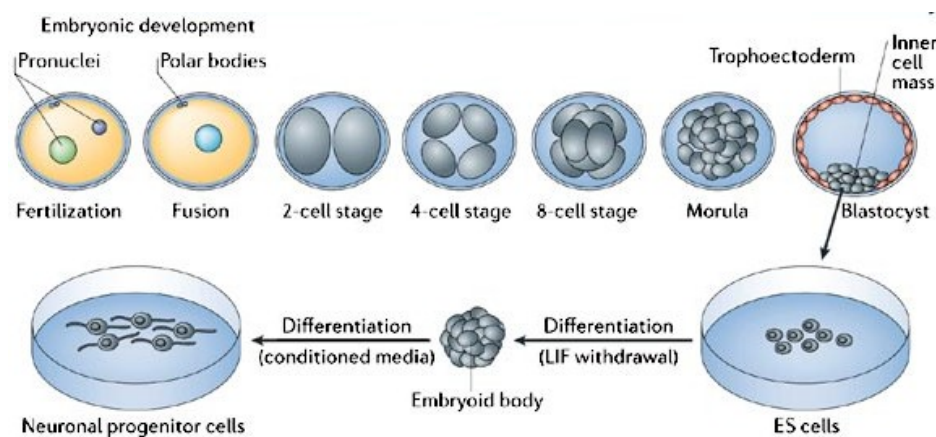


Figure: 1. Embryonic development, embryoid bodies and differentiation.

These spherical structures are comprised of derivatives of all three germ layers. (Wartenberg et al., 1998, Desbaillets et al., 2000). Formation of embryoid bodies can be achieved by removal of feeder cell layer and LIF followed by suspension culture (hanging drop method) or by cultivation in methyl cellulose containing medium (XuC et al., 2001). ES cells have great potential to develop *in-vitro* into all the cell types including neurons (Muotri et al., 2005).

The process of development in which a less specialized cell becomes a more specialized cell type is called differentiation (Figure: 1). As mentioned earlier, ES cells are pluripotent cells, which have the property to divide unlimitedly and remain undifferentiated *in-vitro*, a process

controlled by the combination of various genes working synchronously. The pluripotent nature of mouse ES cells was formally demonstrated by their ability to contribute to all tissues of adult mice, including the germ line, following their injection into host blastocysts (Keller 2005). In addition to their developmental potential *in vivo*, ES cells display a remarkable capacity to form differentiated cell types in culture (Keller 1995; Smith 2001). Over the last 20 years, various studies have led to the development of appropriate culture conditions and protocols to generate a broad spectrum of lineages (Keller, 2005). The close similarity between ES cells and pluripotent embryonic cells implies that they can be used directly to characterize developmental events. Such a model embryo system is required because at the stage when crucial determinative events occur the small size and inaccessibility

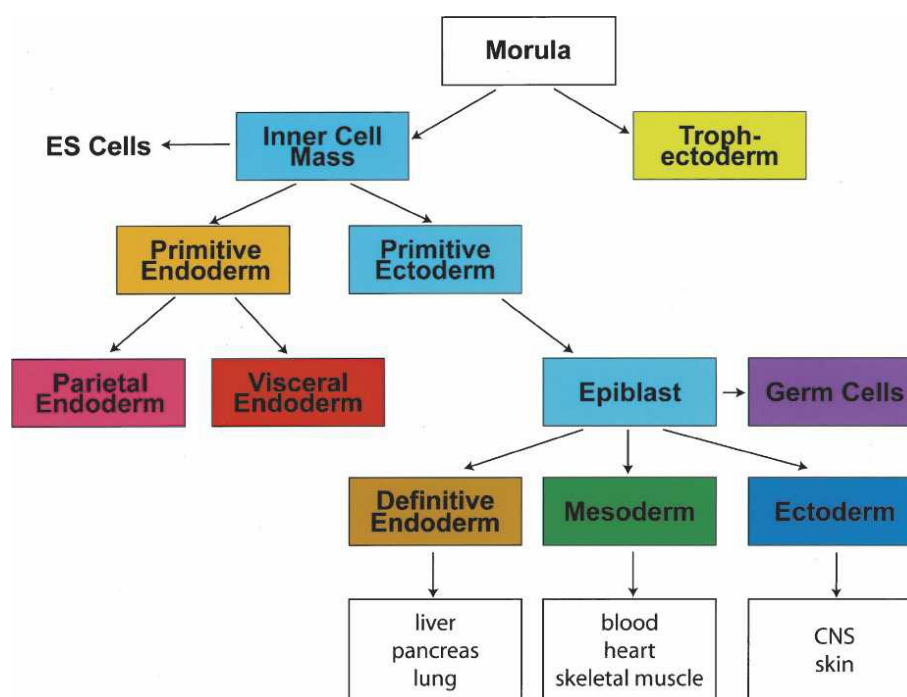


Figure: 2. Schematics of differentiation steps from morula stage to terminally differentiated tissues.

of the mammalian embryo renders it intractable to conventional biochemical analysis (Evans and Kaufman 1981). Because of these unique abilities ES cells may be exploited to identify and define regulatory mechanisms of stem cells and the establishment of differentiated lineages (Figure: 2) (Giguere et al., 1987; Heath et al., 1988). ES cells were firstly cultivated and maintained by co-culturing with mouse embryonic feeder cells (Evans and Kaufman

1981; Martin, 1981). The successful culture of pluripotent cell lines was found to be dependent on three distinct factors – exact stage of isolation of inner cell mass from the embryo, expansion of sufficiently large number of precursor cells from each embryo, and tissue culture conditions which were conducive to multiplication rather than differentiation (Evans and Kaufman, 1981).

Traits that allow ES cells to differentiate into different cell types are regulated by various signalling pathways. Critical components of this include transcription factors, chromatin regulators, histone modifications, signalling molecules and regulatory RNAs (Guenther, 2011). At genetic level the trio of *Oct4*, *Sox2* and *Nanog* forms the core of the ES cell transcriptional regulator network that extends out to other transcription factors and epigenetic modifiers (Boyer et al., 2005; Loh et al., 2006).

1.2) Induced pluripotent stem (iPS) cells

The idea of induced pluripotent stem cells came into existence in order to circumvent the issues of host rejection following transplantation in patients and because of ethical reason regarding human embryos. Induced pluripotent stem cells commonly known as *iPS* cells are pluripotent stem cells that are artificially derived from non-pluripotent cells like somatic cells. Induced pluripotent stem cells are adult cells that have been genetically reprogrammed to an embryonic stem cell like state by manipulating the genetic makeup of cells (Takahashi & Yamanaka., 2006). Somatic cells can be reprogrammed by transferring their nuclear contents into oocytes or by fusion with ES cells (Cowan et al., 2005; Tada et al., 2001), indicating that unfertilized eggs and ES cells contain factors that can confer totipotency or pluripotency to somatic cells. The iPS technology as we know now is a classic example of inter-disciplinarity of biological science. *iPS* came into existence because of advancement in three major streams of research. First reprogramming was carried out by nuclear transfer, which was reported by Gurdon in 1962. In his laboratory they were able to generate tadpoles from unfertilized eggs that had received a nucleus from an intestinal cell of adult frogs (Gurdon et al., 1962). Further

the generation of the first mammal, “Dolly”, by somatic cloning of mammary epithelial cells by Ian Wilmut and colleagues demonstrated that even differentiated cells contain all genetic information that is required for the development of an entire organism (Wilmut et al., 1997). The second major discovery was the idea of “master” transcription factors, such as the discovery of the *Drosophila* transcription factor, Antennapedia (Schneuwly et al., 1987), and of the mammalian transcription factor, MyoD (Davis et al., 1987). And third was the establishment of stable mouse ES cell culture that enabled maintenance of long term pluripotency (Evans and Kaufman 1981; Martin 1981).

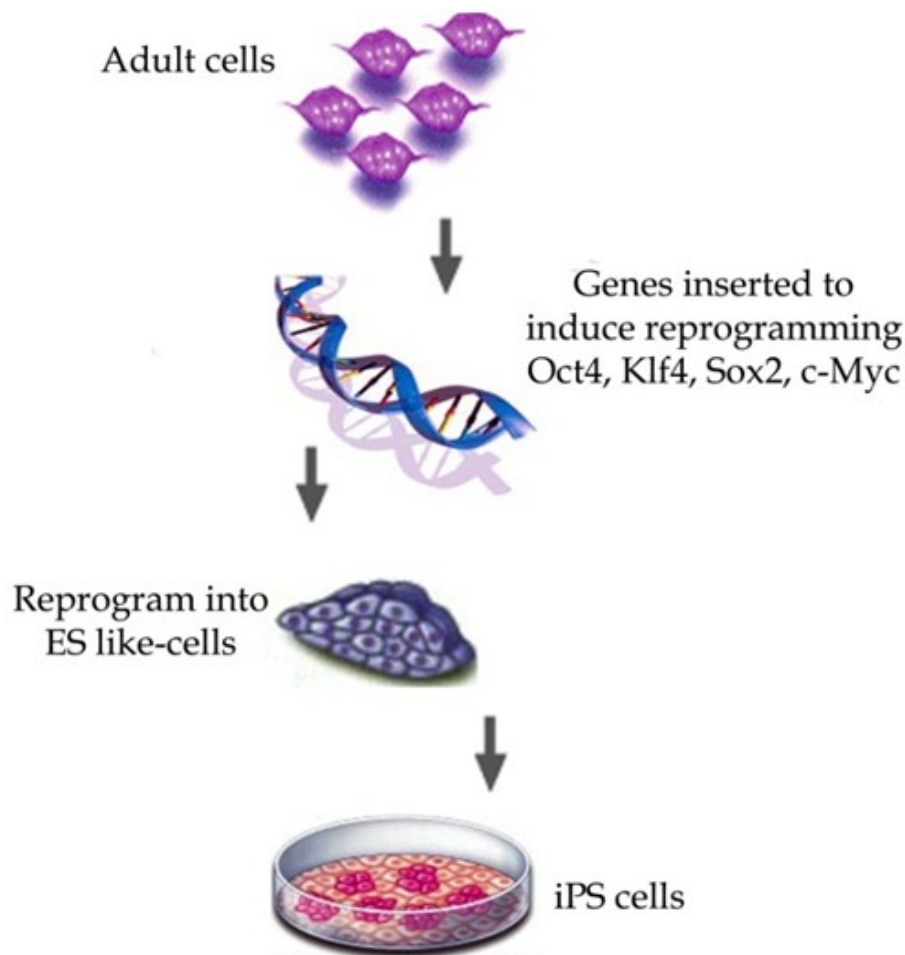


Figure 3. Schematic representation of *iPS* cell formation. (Meregalli et al., 2011)

By combining these three different streams of research it was feasible to hypothesize that a combination of multiple factors in oocytes and ES cells reprogram somatic cells into the embryonic state (Yamanaka et al., 2012). To identify these transcriptional regulators (master

genes) that can reprogram adult cells into pluripotent cells, 24 pluripotency associated candidate genes that can activate a drug resistance allele driven by *Fbxo15* promoter were integrated into the ES cells. The combination of 24 factors, when co-expressed from retroviral vectors in mouse fibroblasts induced the formation of drug resistant colonies with characteristic ESC morphology (Takahashi & Yamanaka., 2006). Successive rounds of elimination of individual factors then led to the identification of a minimally required core set of four genes, comprising *Klf4*, *Sox2*, *c-myc*, and *Oct4* (Figure: 3). *iPS* cells generated by selection for *Fbxo15* activation expressed markers of pluripotent stem cells such as *SSEA-1* and *Nanog*, generated teratomas when injected subcutaneously into immune suppressed mice and contributed to different tissues of developing embryos upon blastocyst injection (Takahashi & Yamanaka., 2006). However, Yu et al. showed that it is also possible to reprogram somatic cells using another cocktail of genes in which *c-myc* was replaced with *LIN-28*, further showing that *Oct4*, *Sox2* and *KLF4* are the key players in reprogramming (Yu et al., 2007).

1.3) Serotonin

1.3.1) History: Discovery of serotonin

Discovery of serotonin is one of those of serendipity. It started with finding the cause for hypertension and ended up being one of the most important discoveries in neuroscience. In the early 1930's, Vittorio Erspamer working in the Institute of Comparative Anatomy and Physiology, University of Pavia, was interested in the smooth muscle contracting properties of various amine substances found in the skins and intestinal tracts of a variety of species, including rabbits, molluscs, and frogs. One substance which interested him was found in enterochromaffin cells of the gut (Whitaker-Azmitia 1999). Dr. Erspamer named it *enteramine* (Erspamer and Villai., 1937). In 1945, Dr. Irvin Page while looking for the substance in the serum causing hypertension brought Maurice Rapport who was an organic chemist and Arda Green an equally talented biochemist together to pursue this research.

Finally the team of Page, Rapport and Green were able to isolate and characterize this serum substance as serotonin. In 1949, Dr. Betty Mack Twarog while studying a phenomenon in mollusc muscle called catch, got into the field of smooth muscle contraction and its relationship with neurotransmitters (which was still highly doubted). In 1951, Dr. Twarog had identified the contracting neurotransmitter in *Mytilus* as acetylcholine, but could not identify the relaxing neurotransmitter. However, over a period of time both Dr. Twarog and Dr. John Welsh were convinced that the missing neurotransmitter is serotonin. The landmark paper that not only cemented serotonin as a neurotransmitter but also decreased the likelihood of its involvement in the development of hypertension was published in June 1953 (Twarog and Page, 1953). However, the final brick in the wall was cemented by the work of Dr. Dilworth Wayne Woolley. His work on Lysergic acid diethylamide (LSD), combined with the findings of Dr. Twarog, was instrumental in recognizing the role of serotonin in the brain. Finally, Dr. Woolley's work and hypotheses were summarized in his book "*The Biochemical Bases of Psychoses or the Serotonin Hypothesis about Mental Disease*" (Woolley 1962).

1.3.2) Distribution of serotonergic neurons in the brain

Serotonin is found in many tissues. The digestive tract contains about 95% of total amount of serotonin of the body, localized in enterochromaffin cells. Practically all blood serotonin (concentration ranging from 10-200 microgram per liter) is found in platelets. However, platelets do not synthesize serotonin, but take it up from plasma into which it is released by enterochromaffin cells. In the CNS of all species serotonin is synthesized in the raphe neurons of the brainstem. The neurochemical anatomy of brainstem serotonin neurons was first studied by histochemical analysis of indole amines by Dahlstrom and Fuxe (Dahlstrom and Fuxe., 1964).

The system Dahlstrom and Fuxe reported, consisted of a relatively small population of morphologically diverse neurons whose cell bodies are present largely within the brainstem raphe nuclei and particular regions of the reticular formation. Raphe clusters of serotonergic

neurons were found rostrally from the level of the interpeduncular nucleus in the midbrain to the level of the pyramidal decussation in the medulla. The raphe nuclei are a collection of neurons, with poorly defined cytoarchitectural limits. They flank midline, along the rostrocaudal extension of the brainstem, both in animals and humans (Meessen and Olszewsky 1949, Taber et al., 1960, Olszewski and Baxter, 1954). They contain a heterogeneous population of neurons with distinct morphologies, projection and neurochemical characteristics. The serotonergic neurons are the major constituents of the raphe nuclei (Hornung, 2003). They develop from mesopontine and medullary primordia resulting in groups of rostral and caudal clusters which are maintained into adulthood and are distinct in their connectivity. Although, there are only about 20,000 serotonergic neurons in the rat brain (around 300,000 in humans) the extensive axonal projection system arising from these neurons bears a tremendous number of collateral branches, so that the serotonergic system densely innervates nearly all regions of the CNS (Figure: 4). The midline raphe nuclei consist of the caudal linear nucleus (CLi, B8), the dorsal raphe nucleus (DR, B6, B7), the median raphe nucleus (MnR, B5, B8), raphe magnus nucleus (RMg, B3), raphe pallidus nucleus (RPa, B1), and the raphe obscurus nucleus (ROb, B2). Outside the raphe nuclei there are collections of 5-HT containing cell bodies in a region adjacent to the medial lemniscus called the B9 cell cluster, in the ventrolateral medulla called the B3 cluster, and in the central gray of the medulla oblongata (B4).

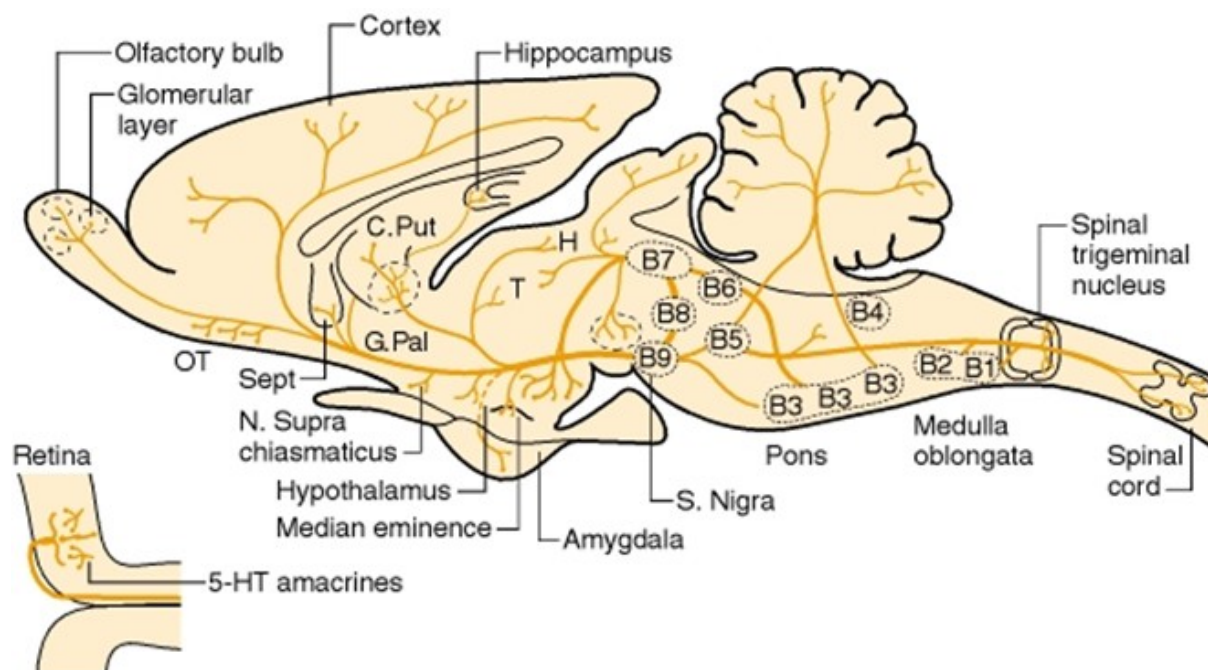


Figure: 4. Serotonergic system and its innervation in CNS. Neurons in the B1-3 groups, corresponding to raphe magnus, raphe pallidus and raphe obscurus nuclei in the medulla, project to the lower brain stem and spinal cord. Neurons in the B4-9 groups, including the raphe pontis, median raphe, and dorsal raphe nuclei, project to the upper brain stem, hypothalamus, thalamus and cerebral cortex. (Basic Neurochemistry: Molecular, Cellular and Medical Aspects. 6th edition, 1999)

The B3 groups of serotonergic neuron clusters are thought to be lateral extensions of the serotonergic neuron clusters in midline raphe (Dahlstrom and Fuxe., 1964).

1.3.3) Chemistry of serotonin synthesis

Biochemically serotonin, also known as 5-hydroxytryptamine (5-HT) is a monoamine. It is derived from tryptophan.

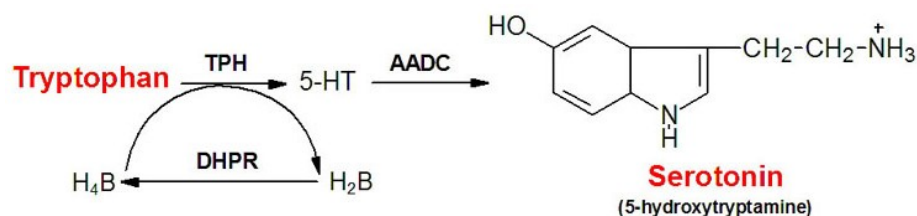


Figure: 5. Synthesis of 5-HT. Serotonin (5-hydroxytryptamine, 5-HT) is formed by the hydroxylation and decarboxylation of tryptophan. TPH, tryptophan hydroxylase; AADC, aromatic amino acid decarboxylase;

The first step is the rate limiting hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP) by tryptophan hydroxylase (TPH) (Figure: 5) that requires Fe^{2+} as a cofactor and O_2 and

tetrahydrobiopterine (BH₄) as co-substrates (Lovenberg et al., 1967). The second step is the immediate decarboxylation of 5-HTP to 5-HT by a nonspecific aromatic amino acid decarboxylase (AADC) (Uchida et al., 1992). Since TPH is the rate limiting enzyme in the synthesis, the rate of formation of serotonin is influenced by the activity of TPH and tryptophan availability.

1.3.4) Metabolism of serotonin

Metabolism of serotonin is carried out primarily by the outer mitochondrial membrane enzyme monoamine oxidase (MAO), which occurs as two molecular subtypes called MAO-A and MAO-B (Cesura et al., 1992). Both subtypes have a widespread occurrence in the brain and in peripheral tissues, although they do show some differences, including species variations, with respect to the extent of their presence in certain tissues and cell types. In addition, the subtypes show differences in their substrate specificities and their sensitivities to certain inhibitors (Chimenti et al., 2010, Shin et al., 1999).

1.3.5) Serotonergic system during development

The serotonergic neurons in the brain are restricted to a brain stem region and to the reticular formation. They are generated around embryonic day (E) 10 to 12 in mouse, but the full maturation of the axonal terminal network requires more time and is achieved only after birth in rodents (Whitaker-Azmitia., 1995). Organogenesis during embryonic development starts after gastrulation and the formation of three germ layers: ectoderm, mesoderm, and endoderm. The specification of serotonergic precursor cells in the neural tube is determined by the combination of secreted factors (Figure: 6B). FGF8 is secreted by the cells at the boundary between the midbrain and hindbrain, in the midbrain-hindbrain organization centre (MHO), FGF4 is produced by the primitive streak located dorsally and laterally in the neural tube; and Sonic hedgehog (SHH) is produced by the notochord in the ventral midline (Figure: 6) (Gaspar 2003). At transcriptional level there is a vivid network of factors controlling 5-HT phenotype in the raphe nucleus. The orientation towards serotonergic lineage happens as early

as the neuroblast stage in the ventricular zone. The key driving factors like FGF4, FGF8, and SHH, activates the expression of Nkx2.2 in the caudal brainstem. Nkx2.2 along with other unknown factors in turn activates the early serotonergic gene, *Lmx1b*, which then turns on its downstream factors like *Pet1*, *Tph*, *Aadc*, *Sert*, and other unknown candidates (Figure: 7).

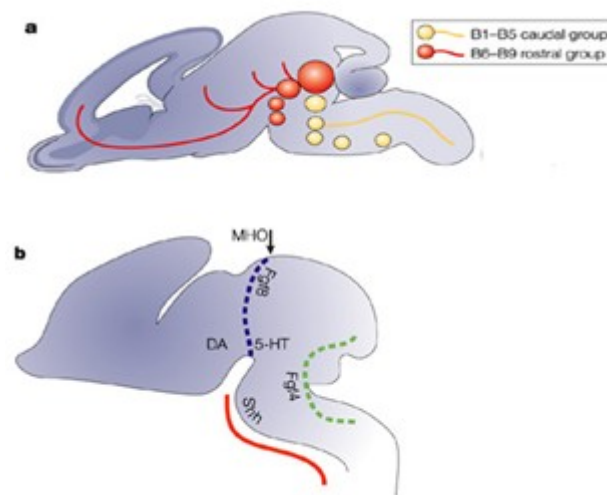


Figure: 6. (a) Organization of serotonergic neurons in brain stem, (b) Factors controlling serotonergic neuron development (Gaspar et al., 2003).

Serotonin has also been detected in embryos before the appearance of neurons. This antecedent 5-HT in early stages of embryogenesis modulates cell proliferation, migration, cell shape and cell-cell coupling. 5-HT has also been previously implicated in the cell cycle and at the same time in apoptosis (Buznikov et al., 2003). This multifunctional role of serotonin comes because of its various receptors which transduce signals into different downstream cellular partners (Hoyer et al., 2002), and because of the two different isoforms of TPH, TPH1 and TPH2, which synthesize 5-HT (Walter and Bader, 2003). 5-HT plays this developmental role in both vertebrates and invertebrates (Buznikov et al., 2005, Xiao et al., 2006, Vesela et al., 2003, Walter and Bader, 2003).

1.3.6) Transcriptional control of serotonergic specification

The transcriptional cascades that are involved in the generation of 5-HT neurons have been identified to a certain extent. The early steps of 5-HT progenitor cell specification, proper development and maintenance of the 5-HT transmitter system requires expression of a variety of proteins that together define the mature phenotype of 5-HT neurons. One of the most important players, which is also the rate-limiting step in 5-HT biosynthesis, is TPH (McGeer and McGeer, 1973). Another well-known factor is the serotonin transporter, SERT, (Blakely et al., 1991) that is involved in serotonin reuptake. These two constitute the most important attributes of the 5-HT producing neuron and their expression is restricted nearly exclusively to 5-HT neurons (Hansson et al., 1998, Rattray et al., 1999, Rind et al., 2000). In addition, the more broadly expressed aromatic L-amino acid decarboxylase (AADC) and vesicular monoamine transporter 2 (VMAT2) are needed for the second and final step of 5-HT synthesis and for packaging of 5-HT in synaptic vesicles, respectively (Weihe and Eiden, 2000). However, the transcriptional controls of these genes are not well understood and transcription factors which form the integral part of the 5-HT cascade are yet to be fully discovered. Nonetheless, the few already identified transcription factors, including *Phox2b*, *Lmx1b*, *Pet1*, *Mash1*, *Nkx2.2*, *Gata2*, and *Gata3*, belong to very diverse groups and have distinct roles in the development and differentiation of serotonergic neuron.

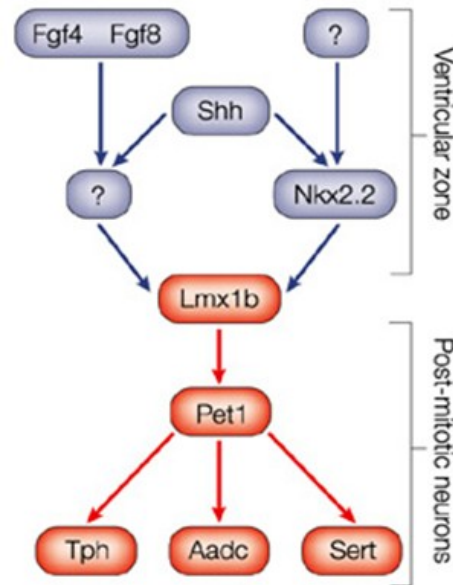


Figure: 7. Transcriptional control of serotonergic neuron differentiation (Gaspar et al., 2003).

Phox2b is a transcription factor belonging to Q50 paired like class (Pattyn et al., 2000) which first represses serotonergic differentiation in rhombomeres r2-r7. Later it is inactivated in r2, r3 and r5-r7 and serotonergic progenitors are produced in the brainstem in different rhombomeres (r1–r3 for the rostral group, r5–r7 for the caudal group) under the influence of a set of secreted factors, including SHH, FGF8 and FGF4, which determine their position in the neural tube. It has been shown that mice lacking *Phox2b* lack all visceromotor neuron precursors and serotonergic neurons are extensively produced in r2-r7, including r4 (Pattyn et al., 2003), confirming the repressing role of *Phox2b* in the development of serotonergic neurons. *Lmx1b*, also known as LIM homeobox transcription factor 1 β is required for the formation of the entire serotonin system in the hindbrain, since its absence in mice results in the lack of serotonergic neurons (Ding et al., 2003, Briscoe et al., 1999, Cheng et al., 2003). It is expressed in the developing serotonergic neurons together with Pet1 starting around E11 in the rostral cluster of serotonergic differentiation and one day after in the caudal one, consistent with the delayed appearance of serotonergic cells in the later region (Cheng et al., 2003). One of the most important players in the differentiation of serotonergic neurons is

Pet1. *Pet1* is an ETS domain transcription factor, which is a specific marker for all the serotonergic neurons from E11 until adulthood (Scott et al., 2005). *Pet1* binding sites are found in the promoter regions of several genes expressed in serotonergic neurons such as *AADC* and *SERT* (Hendricks et al., 2003). Not to surprise, the mice lacking *Pet1* show severe deficiency of serotonergic neurons (about 70% fail to differentiate), whereas in the remaining neurons there was diminished expression of *VMAT*, *TPH* and *SERT* (Hendrick et al., 2003). *Nkx2.2* is another transcription factor which starts to be expressed very early in all serotonergic neuron precursors (about E10.5). Mice lacking this factor do not express *Gata3*, *Lmx1b* and *Pet1* in caudal raphe and no serotonergic neurons develop there (Briscoe et al., 1999, Ding et al., 2003). Out of six Gata (GATA-motif binding) transcription factors that exist in vertebrates, it is shown that at least two, i.e. *Gata2* and *Gata3*, are expressed in the developing brain (Peterkin et al., 2003). Upon deletion of *Gata2*, no 5-HT neurons developed, indicating that *Gata2* also is pivotal for serotonergic differentiation in general. However, mice lacking *Gata3* showed not complete absence but a considerable lack of serotonergic neurons (Van Doornick et al., 1999), showing that also GATA factors are important in the differentiation and development of serotonergic neurons.

1.3.7) In vitro differentiation of ES cells to serotonergic neurons

Different methods have been established to generate serotonergic neurons from undifferentiated ES cells. Possible strategies include the overexpression of known transcription factors such as *Pet1*, *Gata2*, *Gata4* and *Lmx1b*, involved in the serotonergic cascade (Alenina et al., 2006). The focused differentiation of ES cells into a population of cells enriched in neurons, which were also shown to be functionally active, was established long ago (Bain et al., 1995, Fraichard et al., 1995). Nevertheless, the accepted methods of neuronal differentiation employing retinoic acid (RA) lead to production of only few 5-HT positive neurons. Therefore, to produce serotonergic neurons, RA-free protocols were developed, including co-culture systems with bone marrow derived stromal cells (Kawasaki et

al., 2000), VIP and PACAP induction (Cazillis et al., 2004). To date there are four basic methods to generate serotonergic neurons from ES cells; (a) Embryoid bodies (EBs) (Lee et al., 2000) (b) Embryoid body formation including selection and amplification of neural precursor cells (Ying et al., 2003), (c) Co-culture of ES cells with a stromal feeder cell line (Barberi et al., 2003) and (d) Differentiation of ES cells in monolayer culture (Alenina et al., 2006). All these methods are based on the initial culture of ES cells with leukemia inhibitory factor (LIF) and fetal calf serum (FCS) and the omission of serum and LIF already at a very early stage of differentiation. The removal of serum partially blocks the differentiation of ES cells into mesoderm and endoderm (Watanabe et al., 2005), whereas treatment with SHH and growth factors like FGF3, FGF4, and FGF8, at later stages drives the cell to midbrain neuronal fate. Terminal differentiation of post-mitotic neurons appears in N2 or N2B27, serumfree media supporting efficient differentiation and maintenance of neural cells in culture (Bottenstein et al., 1979).

An improved serotonergic protocol, based on overexpression of human *Lmx1b* in ES cells was recently reported. Treatment of neural precursors with the floor plate signal SHH further enhanced the differentiation of *Lmx1b*-overexpressing neural precursors into neurons expressing serotonergic markers (Figure: 8) (Dolmazon et al., 2011).

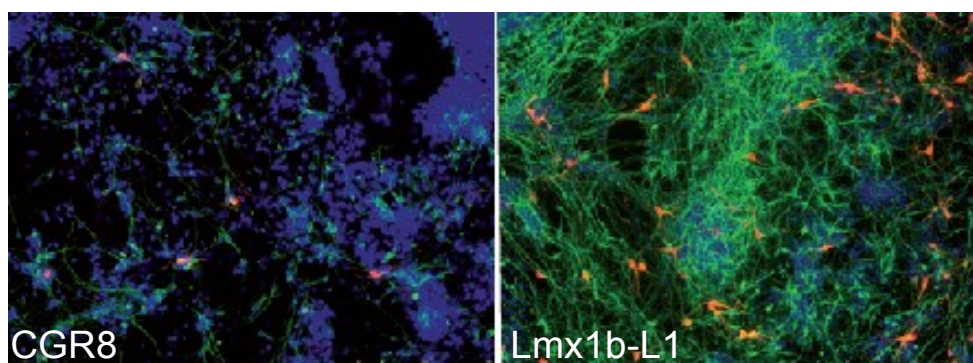


Figure: 8. Serotonergic differentiation of mouse ES cells overexpressing hLmx1b. Double immunofluorescence analysis of Tuj1 (green)- and 5-HT (red)-positive neurons, performed at differentiation day 15 of *Lmx1b* over expressing clone L1 and the parental CGR8 line. Nuclei are labeled with DAPI. Adapted from Dolmazon et al., 2011.

1.4) Zebrafish development

Zebrafish has a number of advantages as a model for studying vertebrate developmental processes. The fish is a vertebrate, and thus, genetically programmed more similarly to that of mammals than invertebrates. This level of similarity is useful, because most organs among vertebrates appear generally similar in form and thus, genes involved in zebrafish organogenesis will likely have human orthologs. In this regard, several zebrafish models of human disease have been characterized (Lieschke et al., 2010).

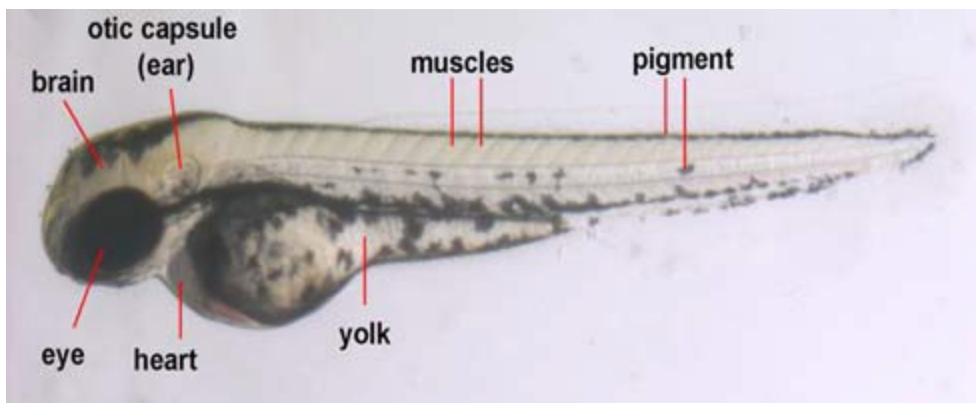


Figure: 9. 2-days old, (48 hours post fertilization (hpf)) zebrafish (*Danio rerio*) (Huang et al., 2005)

The transparent nature of zebrafish embryos enables one to observe individual regions of the brain, heart, and CNS (including spinal cord and notochord) during development. The development of zebrafish is broadly classified into seven different stages. These stages can be briefly described as following (the timing is given in “hour post fertilization” (hpf)).

Zygote Period (0-0.75.hpf): During this stage the cytoplasm streams toward animal pole to form the blastodisc.

Cleavage Period (0.75-2.2 hpf): During this period, the first 6 cleavages occur. The cells, or blastomeres, divide synchronously at about 15 minute intervals.

Blastula Period (2.25-5.25 hpf): Midblastula transition occurs at the 10th cleavage. At this division, cell membranes do not form between cells of the bottom, marginal, row of

blastomeres, and thereafter, it develops into the "yolk syncytial layer (YSL)" of the yolk cell. After midblastula, transition cell divisions are asynchronous. Margin reaches 30% epiboly. Epiboly is described as a cell movement that occurs in the early embryo, at the same time as gastrulation. It is one of many movements in the early embryo that allow for dramatic physical restructuring. The movement is generally characterized as being a thinning and spreading of cell layers.

Gastrula Period (5.3-10 hpf): Around 8 hours margin reaches 75 % epiboly. Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis through the end of epiboly.

Segmentation Period (10-24 hpf): Somites, pharyngeal arch primordia, and neuromeres develop; primary organogenesis; earliest movements; the tail appears.

Pharyngula Period (24-48 hpf): Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development.

Hatching Period (48-72 hpf): Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously. At 72 hpf, swim bladder inflates; food-seeking and active avoidance behaviours.

1.4.1) Central nervous system (CNS) development of zebrafish

The CNS of vertebrates develops from a specialized region of the ectoderm called the neuroectoderm or neural plate, which is specified during the process of gastrulation. Gastrulation is a phase early in the embryonic development of most animals, during which the single layered blastula is reorganized into a three layered structure known as gastrula. These three layers are known as ectoderm, mesoderm, and endoderm. One of the major steps in gastrulation is called neurulation, which involves formation of the neural plate, which is a part of ectoderm. In contrast to other vertebrates, zebrafish neural plate is converted at the beginning into a solid structure, the neural keel, which develops later by dissolution of cells in the centre into neurocoel or central canal (Figure: 10) (Amacher et al., 1998).

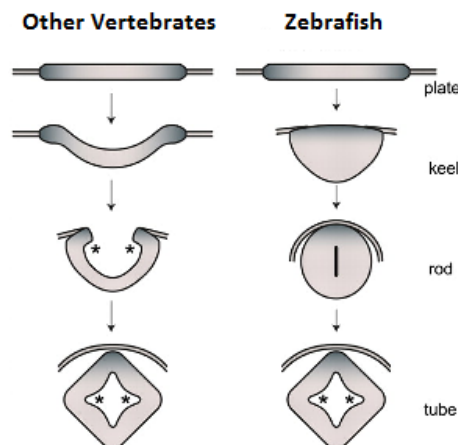


Figure: 10. Formation of Zebrafish neural tube vs. other vertebrate neurulation (Molly et al., 2009).

The neural keel forms in an anterior to posterior progression (Blader and Strehle, 2000, Papan et al., 1994). The first neuron becomes post-mitotic in the neural plate shortly after gastrulation and before the transformation into the neural keel has started (Mendelson 1986). As a consequence, the embryos are already motile by the end of the first day of development, showing spontaneous twitching of the body. During second day of development the embryos start to respond to touch stimuli. This response is coordinated by relatively few differentiated neurons and a simple scaffold of pioneering axons. The neural tubes of zebrafish embryos show a segmental organization. This is most apparent in the hindbrain and spinal cord, but has also been proposed to extend into the anterior brain (Kimmel 1993). Segments or neuromeres are outlined by clusters of neurons in the brain of a 1 day old embryo (Essen 1991). Similarly, primary motor neurons and some interneurons in the spinal cord are arranged segmentally in register with the adjacent somites (Kuwada et al., 1990).

1.4.2) Zebrafish lateral line system

The lateral line is a part of the motor neuron system in zebrafish embryos which acts in response to differences in the motion of water and is involved in a large spectrum of behaviour from prey detection to predator evasion, school swimming and sexual courtship. It

is a sensory system which is exclusively present in fish and amphibians (Stone 1938). It has disappeared in the terrestrial tetrapod, with the exception of its internal counterparts, the inner ear. The lateral line comprises a set of sensory organs, the neuromasts (Figure: 11), arranged on the body surface in species specific patterns. Neuromasts comprise a core of mechanosensory cells, and are innervated by sensory neurons that are localized in a cranial ganglion. The neuromasts on the head form the anterior lateral line system (ALL), the ganglion of which is located between the ear and the eye, while the neuromasts on the body and tail, including those on the caudal fin, form the posterior lateral line system (PLL), its ganglion being just posterior to the ear. The most interesting aspect of lateral line development is that the neuromasts are deposited by the migrating primordium, which originates from cephalic placodes (Stone 1938).

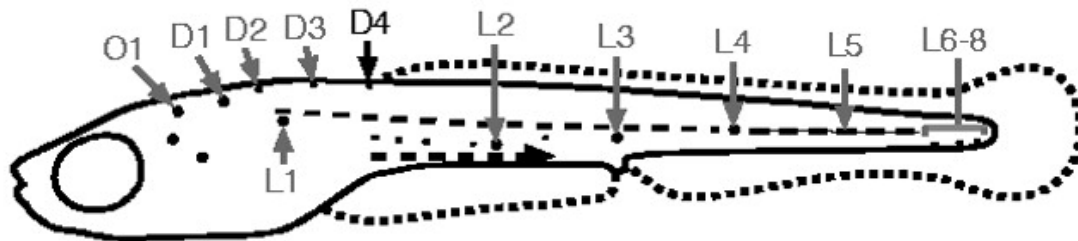


Figure: 11. Arrangement of mechanosensory neuromasts cells in zebrafish lateral line. The neuromast are arranged in a specific manner from head to tail.

Even more interesting is the fact that the PLL primordium follows a pre-existing pathway and the deposition of neuromasts is due to a mechanism that is intrinsic to the primordium, rather than being an exterior response (Smith et al., 1990). The lateral line system of the embryonic zebrafish is highly stereotyped (Raible and Kruse 2000). The neuromasts of the PLL are deposited at regular intervals by the primordium, which originates from a placode that is just posterior to the otic placode, and migrates along the horizontal myoseptum (Metcalf et al., 1985) all the way to the tip of the tail. Each neuromast is made up of hair cells surrounded by support cells. Neuromasts are generally exposed to the body surface, but they may also be

embedded in pits or in sub epidermal canals. They are easily visualized with Nomarski optics or after incubation of live fish with the vital fluorescent marker, 2-Di-4-Asp or DASPEI (Collazo et al., 1994), which accumulates specifically in active hair cells. Neuromasts are innervated by sensory neurons as well as by efferent fibres that control the sensitivity of the system. The sensory neurons are bipolar and extend a central projection into the hindbrain (Dambly-Chaudiere et al., 2003). The migration of zebrafish lateral line primordium is a complex process that involves number of known and unknown gene orchestrating the development of zebrafish. The directed migration of the cell cluster follow the path of the Sdf1a chemokine and requires two receptors, Cxcr4b and Cxcr7b, which are expressed in the leading and trailing part of the primordium, respectively. However, the downstream factors mediating this control are still unclear and remain to be found (Breau et al., 2013, Xu et al., 2014).

2. AIM OF STUDY

With increasing cases of depression in 21st century it is of utmost importance to understand the mechanism behind the pathogenesis of this disease. According to a WHO report published in 2012, depression is not only prevalent in developed countries, but is a significant contributor to disease burden and affects people across the globe. It is estimated to affect about 350 million people around the world (*WHO report: Depression- A Global Public Health Concern, 2012*). So, finding the root of the problem when it comes to depression is extremely important. Several factors seem to contribute to bring about depressive disorders, and serotonin is discussed to play an important role in their genesis. Almost all the corticolimbic structures that are involved in mood regulation and the stress response, such as prefrontal cortex, amygdala, hippocampus, and nucleus accumbens (Holmes, 2008; Steinbusch, 1981) are extensively innervated by axons, originating from dorsal raphe and express receptors for 5-HT. The origin of 5-HT impairment in depression is multifaceted and is likely due to interaction of many intrinsic factors like genetic predisposition, gender and personality factors; and extrinsic factors like stress, environment, and insufficient social support (Jans et al., 2007). Changes in serotonin metabolism may be an important factor in the etiology and treatment of depression (Putin et al., 1992). Selective serotonin reuptake inhibitors (SSRIs) are nowadays the most commonly used drugs for uncomplicated depression. However, drugs only serve to tackle symptoms and rarely the root cause. They also produce many unwanted side effects, often times with deadly consequences. Taking all these points into consideration, a better understanding of serotonergic neuron biology is of high importance to gain new insights into the pathogenic mechanisms of depression. Therefore, **the main aim of the study was to elucidate developmental mechanisms driving differentiation and specification of serotonergic neurons.**

Using gain- and loss- of function studies several genes which are essential for serotonergic differentiation, such as transcription factors *Pet1*, *Lmx1b*, and *GATA2 and 3* (Ding et al., 2003, Cheng et al., 2003, Murphy et al., 2004) were previously identified. However, the picture of regulatory cascades driving the process of serotonergic differentiation is still incomplete. We aimed to find and characterize new genes which might be involved in the process of serotonergic neuron formation.

First, to select candidate genes essential for serotonergic differentiation we took advantage of *in vitro* differentiation systems. This included

1. Establishment of efficient protocols for the *in vitro* differentiation of ES cells to serotonergic neurons
2. Comparative analysis of gene expression profiles of serotonergic *versus* pan-neuronal differentiation.

The second part of the study aimed to evaluate the candidate genes in different *in vitro* models such as rat iPS and mouse ES and iPS cells. This included

1. Generation of mouse and rat iPS cells.
- 2 Their differentiation towards serotonergic phenotype.
3. Evaluation of candidate gene expression at different stages of differentiation.

Finally, the third aim of the work was investigate the role of selected candidate genes in

1. *in vivo* development of the serotonergic system in mouse and zebrafish
2. embryonic development of zebrafish using morpholino-mediated knockdown

3. MATERIALS AND METHODS

3.1) Materials

<u>3.1.1) Chemicals</u>	<u>Company, Country</u>
Acrylamide (40%, 20:1)	Roth, Germany
Agar	Difco, USA
Agarose	Gibco, USA
Ammonium acetate	Sigma, Germany
Ampicillin	Serva, Germany
Bacto-Agar	Difco, USA
Bacto-Yeast-Extract	Difco, USA
Bis-Acrylamide	Serva, USA
Blocking reagent	Roche, Switzerland
Bromophenol blue	Sigma, Germany
BSA	Sigma, Germany
Chloroform	Merck, Germany
Diethyl pyrocarbonat (DEPC)	Sigma, Germany
Dimethyl sulfoxid (DMSO)	Merck, Germany
Dithiothreitol	Sigma, Germany
DNaseI	Boehringer, Germany
dNTP (100 mM)	Amersham, Germany
Donkey Serum	Sigma, Germany
Ethanol	Merck, Germany
Ethidium bromide	Serva, Germany
Ficoll 400	Promega, USA
Formaldehyde	Merck, Germany
Formamide	Fluka, Germany

Glycerol	Sigma , Germany
Hexamers	Gibco , USA
IPTG	Biomol , Germany
Iso-propanol	Roth, Germany
Methanol	Roth, Germany
Nimodipine	Tocris, Germany
Odyssey Blocking buffer	LI-COR GmbH, Germany
Phenol	Sigma, Germany
Penicillin-Streptomycin (10u/ml)	Gibco, USA
Paraformaldehyde	Otto-Fischer, Germany
Reserpine	Tocris , Germany
SDS	Serva, USA
TEMED	Gibco, USA
Triton X-100	Serva,USA
Tris	Roth,Germany
TRIzol reagent	Invitrogen,Germany
Tween-20	Sigma ,Germany
Urea	Sigma, Germany
X-Gal	Biomol, Germany

3.1.2) Enzymes, Markers and Kits**Company, Country**

Alkaline phosphatase	Promega, USA
Collagenase Typ I	Sigma, USA
DNase A	Roche, Germany
DNase I (RNase-free)	Roche, Germany
GoTaq qPCR Master Mix	Promega, USA
MMLV	Invitrogen, Germany
DNA ligase	Gibco, USA
Proteinase K	Invitrogen, Germany
Restriction enzymes	NEB, Germany
RNase A	Applchem, Germany
RNase Inhibitor	EURx, Poland
RNasin	Promega, USA
T4 -DNA-Ligase	Promega, USA
Taq-DNA-Polymerase	Invitrogen, Germany
ΦX174-DNA/ <i>Hae</i> III-Marker	Fermentas, Lithuania
Lambda DNA/ <i>Eco</i> RI+ <i>Hind</i> III (EH) marker	Fermentas, Germany
Quick Load 100bp DNA Ladder	NEB, Germany
Quick Load 1Kb DNA Ladder	NEB, Germany
Precision Blue Protein Standard All	BioRad Lab, Germany
Bicinchoninic Acid (BCA) Protein assay kit	Sigma-Aldrich, Germany
RNeasy Mini Kit	Qiagen, Germany
QIAquick Gel Extract Kit	Qiagen, Germany
Plasmid Maxi Kit	Qiagen, Germany
Transcription Kit	Stratagene, USA

3.1.3) Equipment and Instruments**Company, Country**

8- channel pipette M300	Biohit , Germany
96 well clear microplates	R&D System, Germany
384 well optical plate MicroAmp	AB Bioscience, Germany
Agarose gel electrophoresis chamber	Biometra, Germany
Binocular microscope	Lecia , Germany
Centrifuge 5415D	Eppendorf, Germany
Centrifuge G5-6R	Beckman Coulter, Germany
Cell culture incubator Heracell	GFL, Germany
Cell culture dishes and plates Cellstar	Greiner bio-one, Germany
Cell strainer	BD Falcons, Germany
Cryostat CM3050 S	Leica, Germany
Disposable pipettes Cellstar	Greiner bio-one, Germany
Electronic pipetter Xplorer	Eppendorf, Germany
Electroporator 2510	Eppendroff, Germany
Falcon tubes	Greiner bio-one, Germany
FastPrep 24 instrument	MP Biomedicals, France
FastPrep lysing matrix tubes	MP Biomedicals, France
Fluorescence microscope BZ-9000	Keyence, Germany
Fuji imaging plates BAS-III	Fujifilms, Germany
Inverted microscope DMI6000B	Leica, Germany
Laminar Flow SAFE 2020	Thermo scientific, USA
Luminescent image analyzer LAS-1000	Fujifilm , Germany
Labofuge 400G	Heraeus Instruments, Germany
Membrane filter	Millipore, Germany
Microbalance	Sartorius, Germany

Microplate Reader Infinite M200	Tecan, Germany
MicroSpin G-50 columns	Amersham Bioscience, UK
Microwave 8020	Privileg, Germany
NanoDrop 1000 spectrophotometer	Peqlab, Germany
Odyssey	Li-Cor Bioscience, Germany
PCR tubes	Biozyme, USA
Pasteur pipettes	Roth, Germany
pH Meter pH level 1	WTW, Germany
Pipetboy accu	Integra bioscience, Switzerland
Power supply PowerPac HC	BioRad Lab, Germany
Precision balance 440-43N	Kern & Sohn Gmbh, Germany
PVDF Membranes	Amersham Bioscience, UK
PTC-100 Programmable thermal Controller	MJ Reserch Inc., USA
Real time PCR system 7900HT AbiPrism	Applied Biosystem, Germany
Roller mixer SRT1	Snijders, Holland
Rotable platform Polymax 1040	Heidolph Instrument, Germany
Save-Lock tubes	Eppendroff, Germany
SDS-PAGE gel electrophoresis chamber	BioRad Lab, Germany
Tank blotter	BioRad Labs, Germany
Thermal cycler S1000	BioRad Labs, Germany
Thermomixer 5437	Eppendroff , Germany
Ultrasound sonoplus	Bandelin electroni, Germany
UV transilluminator MultiImage	Alpha Innotech, USA
Vacuum pump Vacusafe comfort	Integra Bioscience, Switzerland
Vortex Genie 2	Bender & Hobein AG, Switzerland
Waterbath	GFL, Germany

Whatman paper (3 mm)

Whatman , USA

3.1.4) Oligonucleotides

All oligonucleotides were synthesized by Biotez Berlin Buch GmbH and delivered in a lyophilized state. The primers were diluted in water to a concentration of 50 pmol/μl and stored at -20°C. For PCR reactions, primers were diluted to a final concentration of 5 pmol/μl in ddH₂O and kept at -20° C.

Mouse Real time Primers

Itm2b

5'- ACAGAGGAGAGCGTGGTGTT -3'

5'- AGAAGGCTCGTTCAGGATGA -3'

Cacna2d1

5'- CATAACATGGACGCCTGTC -3'

5'- GCACCAGGTCCACTTTTGTT -3'

Ccdc3

5'- GCAGTACCAAGCTGGAGAGG-3'

5'- GTTCTCCTGGGTGTCTGGAA-3'

Lgi3

5'-ATTGGAGGTGGTCTGTTTGC-3'

5'-ATGAAGACGGTGCAGATTCC-3'

Islr2

5'-CCCTGAGTCTTTCAGCGAAC-3'

5'-ATTTGGGCATGCTAGTGACC-3'

Nanog

5'-CTGAACCTGAGCTATAAGCAG-3'

5'- ACCA T/C TGGTTTTTCTGCCACC-3'

Nat1

5'-ATTCTTCGTTGTCAAGCCGCCAAAGTGGAG-3'

5'-AGTTGTTTGCTGCGGAGTTGTCATCTCGTC-3'

TBP

5'-CCCTATCACTCCTGCCACACC-3'

5'-CGAAGTGCAATGGTCTTTAGGTC-3'

Rat RT-PCR Primers*Gata4*

5'-GCATCCATTTCCACCTCTT-3'

5'-TCCATCACCCCTTGTCCTTT-3'

AFP

5'-GTCCCACCCTTCCACTTT-3'

5'-CCATCCTGTAGGCACTCC-3'

FLK

5'-ATACACCTGCACAGCGTACAG-3'

5'-TCCCGCATCTCTTTCACTCAC-3'

Sox17

5'-AGGAGAGGTGGTGGCGAGTAG-3'

5'-GTTGGGATGGTCCTGCATGTG-3'

Nestin

5'-AGCCATTGTGGTCTACTGA-3'

5'-TGCAACTCTGCCTTATCC-3'

NCAM

5'-TGCTCAAGTCCCTAGACTGGAACG-3'

5'-CTTCTCGGGCTCTGTCAGTGGTGTGG

Zebrafish RT-PCR Primer*Cacna2d1*

5'-TCAGAAGATCACTGCCAACG-3'

5'-TGAGATCCTTGGGTTGAAGG-3'

TBP

5'-ATCGGTTTGTGTTGGCTGTCC-3'

5'-TGACCAACAGTACGAGGACC-3'

For zebrafish in situ hybridization the same primer pairs was used for RT-PCR, were used to synthesize in-situ probes.

3.1.5) Zebrafish Morpholinos

Morpholinos were ordered from Gene Tool LLC, Philomath, OR 97370 USA. The oligos were delivered as pre-quantified, sterile, salt-free, lyophilized solid in a glass vial. *Cacna2d1* morpholino was dissolved in 30 mL of sterile water to get a resulting concentration of 1.0 millimolar (mM). The oligos were further diluted to working concentration (1 mM). For injection, about 1 to 10 nanoliters of 1mM oligo was used. Following morpholino sequence was used for injection:

Cacna2d1 : GTGTTTGTTTGTGTACTTACAGCCC

3.1.6) Antibodies

Primary antibodies

Name	Host	Dilution	Company
Oct4	Mouse	1:500	Santa-Cruz Biotechnology
SSEA-1	Mouse	1:500	Developmental Studies Iowa
Nanog	Mouse	1:500	COSMO BIO Co
Class III β tubulin	Mouse	1:500	Covance
Cacna2d1	Mouse	1:100	AbCam
5-HT	Rabbit	1:1000	Sigma
Tuj1	Mouse	1:500	Berkeley

Secondary antibodies

Cy3 anti-mouse	Donkey	1:1000	Jackson Immuno Research
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Alexa 488 Goat 1:1000 Invitrogen

3.1.7) Cell culture media

Cell culture media used in the differentiation and maintenance of ES cells:

MEF/REF medium: For mouse and rat embryonic fibroblasts (MEF and REF, respectively) high glucose DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1x non-essential amino acids, 50 µM β-mercaptoethanol. MEFs were inactivated by cultivation in cell culture medium supplemented with 10 µg/ml mitomycin C for 2 hr.

ES medium: Knockout DMEM supplemented with 15% ES cell-qualified fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1x non-essential amino acids, 50 µM β-mercaptoethanol.

KSR medium: Knockout DMEM supplemented with 10% KnockOut serum replacement, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 1x non-essential amino acids, 50 µM β-mercaptoethanol.

N2B27 medium: Mixture 1:1 of N2-media (DMEM/F12 supplemented with 1x N2, 100 U/ml penicillin, 100 µg/ml streptomycin, 0.005% BSA, 25 µM β-mercaptoethanol) and B27 media (NBM/Neurobasal medium, supplemented with 1x B27 (without RA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 25 µM β-mercaptoethanol).

miPS medium: Knockout DMEM with 10% FBS, 10% Knock-out serum replacement (v/v), 1X L-glutamine, 1% P/S, 1X nonessential amino acids, 100 µM β-mercaptoethanol.

List of supplements used in cell culture media:

Supplements	Final Concentration	Company
LIF	500 U/ ml	Chemicon, Germany
CHIR99021	3 μ M	Stemgent, USA
PD0325901	1 μ M	Stemgent, USA
A-83-01	0.5 μ M	Tocris Bioscience
SHH (Sonic Hedgehog)	10 μ g / ml	R&D Systems, Germany
FGF4	10 μ g / ml	R&D Systems, Germany
FGF2/ bFGF	10 μ g / ml	R&D Systems, Germany
Ascorbic acid (AA)	10 μ g / ml	Sigma, Germany
Retinoic acid (RA)	1 mM	Sigma, Germany
Doxycyclin	10 μ g / ml	Invitrogen, Germany

3.1.8) Cell lines**1) *E14Tg2a* (Bay Genomics, San Francisco, CA)**

E14Tg2a is ES cell line available from Bay Genomics and shared among the members of FunGenES consortium for standardizing conditions and comparing the results of ES cell differentiation performed in different labs.

2) *Parental CGR8 ES cell line and Lmx1b overexpressing line Lmx1bL1*

The cell lines were kindly provided by Pierre Savatier, Institut National de la Sante et de la Recherche Medical (INSERM), Paris, France.

3.1.9) Mouse and zebrafish strains

1) *Cacna2d1* mouse: *Cacna2d1* knockout mouse brains were provided by the group of Dr. Annette Dolphin, from UCL, London, UK (see introduction for details).

2) *Tph2::eGFP*^(FRT-neo-FRT) mouse: The transgenic mouse was generated in the group of Dr. Massimo Pasqualetti at University of Pisa, Italy. We received *Tph2::eGFP*^(FRT-neo-FRT) mouse

embryonic fibroblasts, which were cultured using the providers protocol (Pasqualetti et al., 2013).

3) **Zebrafish strains:** Both wild type zebrafish (Zoltan E strain) and tg(cldnB:lyn2GFP) strain were provided by the group of Dr. Salim Seyfried, MDC, Berlin Buch, Germany.

3.2) Methods

3.2.1) Cell culture and cell biology

3.2.1.1) Derivation of rat iPS cells

To isolate REFs (Rat embryonic fibroblasts) naturally mated Fischer344 female rats were checked for the presence of a vaginal plug and thereafter sacrificed at day 14 of pregnancy (E14). Isolated embryos were freed from head and visceral tissues, minced, subsequently trypsinized and plated on tissue culture dishes (passage 0). REFs at passage 3 were seeded at 1.5×10^4 cells/cm² in a 6-well plate and transduced 24 hrs later with the LVTHM-based Oct4, Sox2, Klf4, cMyc, and EGFP lentiviruses (Wiznerowicz et al., 2003). Four days later cells were split onto a 10 cm culture dish, containing mitomycin-inactivated MEFs and cultured in one of the three reprogramming media (ES, KSR, N2B27 + supplements). After 10-12 days, riPS cell colonies were picked, dissociated in TrypLE-express, and expanded on MEF-feeder cells in the respective reprogramming medium.

3.2.1.2) Maintainance of riPS and electroporation

riPS cells were routinely maintained on mitomycin-treated MEFs in N2B27+2i+LIF media. Cells grew as round, compact colonies, which tended to detach after 2-3 days in culture. Cells were routinely replated every 3–5 days using TrypLE-express. Depending of the proportion of attached cells, riPS cells were either trypsinized on the plate, or collected from the supernatant, centrifuged, and dissociated in suspension. This procedure allowed removal of dead feeder cells as well as differentiated riPS cells, which were usually stably attached to the substrate. For stable transfection, riPS cells were harvested with TrypLE-express, and

subsequently co-electroporated (5×10^6 cells, 240 V, 500 μ F) with 25 μ g of circular *pMC-Cre* (kindly provided by K. Rajewsky) and linearized *p2A2B-tk-TKiresPuro* plasmids (7:1 molar ratio). Puromycin (2 μ g/ml) was added 48 hrs later for 3 days; clones were picked 10 days after electroporation into 96-well plates and genotyped by PCR, using primers specific for exogenous *Oct4*, *Klf4*, *Sox2*, *cMyc*, and *EGFP* and for the *2A2B-tk-TKiresPuro* cassette. Three sub clones, derivatives of the clone IIB9 were chosen for further analysis. riPS cells harboring the *2A2Btk-TKiresPuro* cassette were occasionally cultured in the presence of 1 μ g/ml puromycin (usually for 3 days every 4–5 passages).

3.2.1.3) Clonability assay

To evaluate the effect of different small molecules on maintaining the self-renewal riPS cells were trypsinized into single cells and seeded at the density of 50 cells/cm² in a 6-well plate on mitomycin-treated MEFs. Cells were cultured for 5 days in N2B27 media containing different combinations of inhibitors and subsequently fixed and subjected to the alkaline phosphatase (AP) staining. Each condition was done in triplicate. The number of AP-positive colonies for each condition was averaged from 3 wells to evaluate the percentage of undifferentiated clones. To assess the viability the total number of survived colonies per well was counted.

3.2.1.4) In vitro differentiation of riPS cells

The *in vitro* differentiation of riPS cells was carried out by the “hanging drop” method. riPS cells were dissociated by TrypLE express, resuspended in N2B27 medium containing CHIR99021, and plated in hanging drops (800 cells per 20 μ l drop). Embryoid bodies, formed in hanging drops were collected after 2 days in 10 ml of N2B27 (w/o CHIR99021) and additionally cultured for four more days in low-adhesive bacterial Petri dishes. Medium was changed every second day. After 6 days, embryoid bodies were harvested and transferred onto Matrigel-coated dishes, approximately 10–12 embryoid bodies per 1-well of a 6-well plate in N2B27 medium. 6–8 days later differentiated cells were either fixed in 4% paraformaldehyde

in PBS and analysed further by immunocytochemistry or used for the RNA extraction and subsequent RT-PCR analysis.

3.2.1.5) Mouse iPS cells

Mouse embryonic fibroblasts (MEFs) containing a GFP allele targeted to the endogenous TPH2 locus (Pasqualetti., 2013) were made from E13.5 embryos and were cultivated in MEFs medium. For iPS cells generation MEF were seeded at 1.5×10^5 cells per well of 6-well plates. Transduction was performed the next day (day 0) with concentrated replication incompetent pseudotyped lentiviruses that expressed reprogramming factors (Oct4, Sox2, cMyc, Klf4) and reversed transactivator in MEF medium. 16-24h after transduction medium was changed to fresh MEFs medium containing 2µg/ml doxycycline (DOX). On day 3, cells were reseeded 1:10 on 10 cm dishes containing mitotically inactivated MEFs in miPS medium with 1000 U/ml LIF and DOX. Fresh ES medium with DOX was added every 48 hours until day 10. Colonies with ES-like morphology were picked at days 10-15 and routinely cultivated in ES-medium without DOX on gelatine-coated dishes.

3.2.1.6) Neuronal differentiation of mouse ES and iPS cells

To differentiate mouse ES and iPS cells to neurons, the embryoid bodies (EBs) protocol was used (Alenina et al., 2006). Cells were cultured till 70% confluency in ES + LIF medium. 24 hour before the formation of EBs, the cells were passaged. The cells were trypsinized and re-suspended in KSR medium. After counting, approximately 6×10^6 cells were plated into a 10 cm non-adherent dish and cultured in KSR medium without LIF for 2 days. On Day 2 of differentiation the cells were carefully transferred to a fresh falcon tube and left for 5-10 min to allow all the EBs to settle down in the tube. The EBs were then re-suspended in KSR medium and returned back to culture in a 10 cm petri dish. On day 4, the medium was changed in the similar way. On Day 6 of differentiation, the medium was replaced with N2B27 medium. On day 8, EBs were collected and allowed to settle down. They were then washed with PBS. After washing step, EBs were trypsinized by adding 0.5 ml of 4X trypsin

and kept in water bath for 2-3 minutes till the EBs were dissociated into single cells. The trypsin was inactivated by adding 5 ml of KSR medium and pipetted up and down thoroughly. After counting the cells were centrifuged and re-suspended in N2B27 medium supplemented with FGF2. These cells were then plated on PDL/Laminin coated plates. On day one post plating (Dpp1), the medium was changed to N2B27 with FGF2. On Dpp2, the medium was changed to N2B27, without FGF2. The culture was maintained in the same medium for desired amount of time in the presence of ascorbic acid (AA). To optimize the condition RA or SHH and FGF4 were added on day 4 (EB4).

3.2.2) Molecular Biology

3.2.2.1) DNA analysis

3.2.2.1.1) Isolation of plasmid DNA from *E.Coli*

Mini Preparation

5 ml of sterile LB medium (with 50 µg/ml ampicillin) was inoculated with a single *E.Coli* colony and incubated overnight at 37 °C, with shaking. 1 ml of this overnight grown culture was centrifuged at 14000 rpm for 1 min. The resulting pellet was then re-suspended in 250 µl of solution E1. Cells were lysed by adding 250 µl of solution E2. Equal amount of solution E3 was added to the tube, and mixed immediately by inverting. The mixture was then left to incubate on ice for 30 min. After 30 min, cell debris and chromosomal DNA were pelleted by centrifugation at 14000 rpm at room temperature for 5 min. The supernatant was then transferred into a new tube and 500 µl of 99% isopropanol was added and the resultant solution was mixed by inverting several times. The sample was then kept to incubate at 10 °C at room temperature. It was followed by 15 min of centrifugation at the highest speed. After centrifugation, the pellet was washed with 70% ethanol, air dried pellet was then re-suspended in 30 µl TE buffer. 5 µl of DNA was taken for the further digestion. DNA was stored at -20 °C for long term use.

Maxi preparation

The plasmid DNA was isolated from 300 ml of overnight culture using Qiagen Plasmid Maxi Kit according to manufacturer's protocol. The DNA was usually dissolved in 200-500 µl of TE buffer and kept at -20 °C for long term.

E1 solution	50mM Tris-HCl pH 8.0
	10 mM EDTA pH 8.0
	100µg/ml RNaseA

E2 solution	200 mM NaOH
	1 % SDS

E3 solution	3 M Potassium Acetate pH 5.5
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3.2.2.1.2) Determination of nucleic acid concentration

The DNA and RNA concentration were determined by OD₂₆₀ compared to OD₂₈₀ using a Nanodrop photometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. A ratio of ≈ 2.0 was accepted as pure for RNA.

When $OD_{260} / OD_{280} > 1.7$, the protein concentration can be neglected and the concentration of nucleic acids was determined by the following ratio :

DNA	$OD_{260} = 1 = 50 \mu\text{g/ml}$ for double stranded DNA
RNA	$OD_{260} = 1 = 40 \mu\text{g/ml}$

When $OD_{260}/OD_{280} < 1.7$ the protein concentration of the solution is too high and required a new phenol extraction of nucleic acids, after which the concentration was re-measured. For long term storage DNA was diluted in TE buffer and kept at -20° C. RNA was diluted in

DEPC water (Diethylpyrocarbonate water) or Rnase- Free water from QIAGEN, and kept at -80° C.

3.2.2.1.3) DNA cloning

Preparation of competent cells

LB Medium (100 ml) was inoculated with a single colony of *E.coli* (strain DH5 α) and the culture was grown at 37 °C overnight. Next day, bacteria were centrifuged (10 min, 4°C, 3000 rpm) and the resulting pellet was resuspended in 50 ml of sterile 50 mM CaCl₂ solution (4 °C) and incubated on ice for 30 min. The bacterial suspension was centrifuged at 3000 rpm for 10 min at 4 °C and the pellet was resuspended in 10 ml of sterile 50 mM CaCl₂ at 4 °C with 15% glycerol. The mixture was dispensed into aliquots of 80 μ l and stored at -80 °C.

DNA fragment ligation and transformation of bacteria

The ligation of an insert into a vector was carried out using the following reaction mix (all the steps were performed on ice).

T-Vector (pGEM, T-Easy Vector)	1 μ l
10x ligation buffer	1 μ l
T4 DNA Ligase (5U/ μ l)	1 μ l
Vector DNA	200 ng

The volume was equated to 10 μ l by adding ddH₂O. The ligation was carried out at 16 °C overnight in a heating block. For the transformation the bacteria were gently mixed with competent bacteria (30 μ l) with 2 μ l of the ligation reaction or with 50 ng of pure plasmid DNA. The reaction was placed on ice for 30 min. In the meantime, LB plates were placed at 37 °C to warm up. The cells were then heat shocked at 42 °C for 90 sec and placed on ice for 2 more min. In order to accelerate bacteria growth, 850 μ l LB medium was added to the reaction which was incubated at 37 °C for 60 min with shaking. After incubation, an aliquot of 150 μ l was spread on an Amp/X-Gal/IPTG plate. The remaining cells were shortly centrifuged, dissolved in 100 μ l LB medium and spread on another Amp/X-Gal/IPTG plate.

The plates were incubated overnight at 37 °C. The selection for the presence of the lacZ gene was carried out by the usual blue white screening method (Sambrook et al., 1989).

3.2.2.1.4) Separation of nucleic acids on agarose gel and DNA purification

DNA molecules can be separated by length using 1-3% (w/v) agarose gels. DNA is mixed with 0.1V of 10X loading buffer, applied on agarose gel chambers and electrophoresed at 1-8 V/cm. The gels are prepared with 0.5 µg/ml ethidium bromide for visualizing DNA under 300nm UV light. The size and the appropriate concentration of the DNA bands can be determined by comparison with standardized molecular weight markers DNA/*EcoRI* + *HindIII* and ϕX174 DNA/*BsuRI* (*HaeIII*). Bands were cut out and purified using Promega DNA purification kit.

3.2.2.1.5) Polymerase chain reaction (PCR)

The PCR conditions were optimized and performed according to general rules.

DNA	2 µl
PCR buffer (10x)	5 µl
Forward Primer (5µM)	2µl
Reverse Primer (5µM)	2µl
dNTPs (5mM)	2µl
MgCl ₂ (50mM)	2µl
<i>Taq</i> Polymerase (1U)	0,3µl
ddH ₂ O	34,7µl

Reaction components for PCR

The reaction mixture was pipetted into PCR tubes and subjected to the following program.

Step	Time	Temperature	Cycles
Initial Denaturation	5 min	95°C	1 x

Denaturation	30 sec	95°C	
Annealing	45 sec	56 - 65°C	35 x
Elongation	30 sec	72°C	
Final Elongation	5 min	72°C	1 x

2-5 µl of PCR reaction was mixed with DNA loading dye and analyzed on 1-2% agarose gel.

3.2.2.1.6) Sequencing

Sequencing of DNA samples was performed by the company Invitex (Berlin Buch) using the BigDye® Terminator v1.1 Cycle Sequencing Kit (Invitrogen) and the 3130 Genetic Analyzer (Applied Biosystems).

3.2.2.2) RNA analysis

3.2.2.2.1) RNA isolation

3.2.2.2.1.1) RNA isolation from cell culture

RNA from cultured cells was purified using the RNeasy® Mini Kit (Qiagen) and manufacturer's instructions were followed. In detail, cells grown in a monolayer ($< 5 \times 10^6$) were lysed by re-suspending them in 350 µl buffer RLT (containing 10 µl β -mercaptoethanol per 1 ml of buffer) using a pipette. 350 µl of 75% ethanol were added, lysates were homogenized by passing them 5 times through a 20-gauge needle and transferred into QIAshredder spin columns placed in 2 ml collection tubes. After centrifugation for 15 sec at 8,000 g, flow through was discarded and columns were washed with 700 µl buffer RW1, followed by another two washes with 500 µl buffer RPE. Columns were then placed in new collection tubes, dried by centrifugation for 1 min at full speed and placed in new 1.5 ml collection tubes. 30-50 µl RNase-free water were added directly to the spin column membrane and samples were centrifuged again for 1 min at 8,000 g to elute the RNA. RNA concentration was measured using the NanoDrop™ spectrophotometer (PepLab) and samples were stored at -80°C.

3.2.2.2.1.2) Isolation of RNA from zebrafish

The embryos were collected at different time points during their development. About 30 embryos were collected at 8 hour post fertilization (hpf), Day 1, Day 2, Day 3, Day 4, and Day 5. The embryos were then pooled into a 1.5 ml Eppendorf tube and crushed manually using a pestle. The RNA was isolated using phenol: chloroform solution (1:1). The RNA was precipitated by adding 0.5 ml of isopropanol. Finally, the pellet was washed with 70% ethanol, dissolved in 80-100 µl of DEPC H₂O. RNA was kept at -20°C. The concentration and purity of isolated RNA was then measured using nanodrop.

3.2.2.2.2) Reverse Transcription (RT)

The preparation of cDNA from DNaseI pretreated total RNA was performed as follows: 2-5 µg of total RNA was mixed with 5 µl of random hexamer primers (20 µM) in a volume of 15 µl DEPC H₂O. To avoid formation of RNA secondary structures the mixture was heated to 65°C for 5 min, and then quickly chilled on ice. After a brief centrifugation, the following master mix was added to the reaction mixture:

5x First strand buffer	6 µl
(200 mM NaCl, 50mM Tris-Cl (pH 8.9), 25mM MgSO ₄)	
0.1 M DTT	3 µl
5 mM dNTPs	3 µl
RNasin (10 U/µl)	1 µl
M-MLV (200 U/µl)	2 µl

All the components in the reaction mixture were mixed gently and centrifuged shortly and incubated at 37°C for 1 hour. Afterwards the reaction was inactivated by heating the samples at 80°C for 10min. 2-3µl of the cDNA solution was used for real-time PCR quantification.

3.2.2.2.3) Real time PCR

Real-time PCR is an efficient and highly sensitive method for relative quantification of gene expression. Real time PCR methods are based on the principle of sequence-specific DNA probes consisting of oligonucleotides that are labelled with a fluorescent reporter and a quencher (TAMRA/FAM) which permits detection of hybridized probe with its complementary DNA target. The degradation of the fluorescent probe by 5' to 3' exonuclease activity relieves the quenching effect and allows the fluorescence emission measured via FRET. The fluorescence detected in the real-time PCR is directly proportional to the fluorophore released and the amount of cDNA template present in the PCR. The real-time PCR approach used the SYBR green reagent. An established method was applied to compare gene expression levels between groups, using the equation $2^{-\Delta\Delta CT}$. Gene expression was normalized to Nat1 mRNA expression. Real time PCR was run in technical duplicate always.

3.2.2.2.4) Preparation of riboprobes for in situ hybridization

The DNA fragments for zebrafish RNA probes (nucleotides 300-600 bp) were obtained by RT-PCR using the total RNA isolated from 3 day old zebrafish embryo as a template and inserted into the pGEM-T or pGEM-T easy vector (Promega). Purified plasmid were then linearized by restriction enzymes digestion, and in vitro transcription was performed with T7 or SP6 RNA polymerase, in the presences of digoxigenin (dig) - UTP (Roche). To purify the ribo-probe, we used a kit (Clean up RNA, QIAGEN).

3.3) Protein biochemistry

3.3.1) Isolation of protein from Zebrafish

The embryos were collected at different time points as mentioned earlier. For isolation of protein, 50 embryos were pooled together at different time points. Pooled zebrafish were dissected under binocular microscope in three different parts; head, trunk and tail tip. Whole protein was isolated using RIPA buffer. The embryos were pooled together in a 1.5 mL eppendorf tube and then 200 μ L of RIPA buffer was added and sonicated (5 cycles + 30 sec,

FWD, 100 %). The solution was then spun at 14000 rpm for 10 min at 4 °C and supernatant was transferred into a new Eppendorf tube. Protein concentration was measured using BCA. The isolated protein was stored in the electrophoresis buffer at -20 °C.

RIPA Buffer	
NaCl	150 mM
Tris-HCL pH 7.5	50 mM
NP-40	1 %
DOC (Natriumdeoxycholat)	0.5 %
SDS	0.1 %
PMSF	1 mM
Complete Protease	
Inhibitor Cocktail	1 Tablet / 10 ml

3.3.2) Western blot

The dissolved embryos were loaded in a SDS gel, run at 20mA per gel until the dye front came close to the bottom, after that the protein were transferred to a nitrocellulose membrane at 100 V for 1 hour 45 min in transfer buffer. The blotting membrane was then incubated first with blocking odyssey blocking buffer bought commercially, for one hour at room temperature (RT) and next incubated with primary antibody diluted in odyssey blocking buffer, overnight. Next morning the blot was washed thoroughly in TBS containing 0.1% Tween 20 with shaking. After that the blot was incubated with secondary antibody for 1 hour at RT and again washed 3 times with TBS for 10 min each. The blots were finally washed with TBS and observed under Odyssey Li-Cor Bioscience reader.

Transfer buffer	
Tris	25 mM
Glycin	200 mM
Methanol	20%

TBS

Tris	50 mM
NaCl	150 mM
pH 7.5	

3.4) Histological and immunofluorescence analysis**3.4.1) Immunofluorescence staining for cell culture**

Immunocytochemistry of undifferentiated and differentiated iPS cells was performed using previously stated protocol (Liskovych et al., 2012) with primary antibodies against Oct4 (1:100), SSEA1 (1:200), SSEA4 (1:100), albumin (1:100), β III-tubulin (1:250) followed by goat anti-mouse IgM-FITC (1:200), chicken anti-goat IgG-FITC (1:200), goat anti-mouse IgG-FITC (1:200), Alexa Fluor 594 goat anti-rabbit IgG (1:750) secondary antibodies. Nuclei were stained with Hoechst 33258.

3.4.2) Mouse tissue immunofluorescence staining

5 μ m thick paraffin sections were deparaffinised with xylol (3 X 5) followed by rehydration in a series of alcohol solutions (2x 100%, 95%, 80%, 70%, 50%, 30%), each step for 5 mins. After two final washes with distilled water and PBS, sections were treated either with citrate puffer (pH 6) for 20 min at 95°C or with trypsin solution (1 mg/ml trypsin in H₂O) for 15 min at 37°C for antigen retrieval. The section were washed (2 X 10 mins) with PBS.

Cryosections were cut and dried overnight at room temperature and were stored at -20°C until ready for use. They were incubated in PBS (pH 7.4) for 20 min for rehydration followed by wash in PBST (0.1% Triton-X 100 in 1x PBS) for 30 min for permeabilization.

Citrate buffer

Citric acid	6.0 mM
Sodium citrate	41.5 mM
ddH ₂ O	

Pre-treated paraffin or cryosections were blocked for 30 min with 10% normal donkey serum in PBS followed by incubation with the primary antibody (diluted in PBS) overnight at 4°C in a humidified chamber. The next day, sections were washed with PBS three times for 15 min and incubated with the secondary antibody (diluted in PBS) for 2 h at room temperature in the humidified chamber. Sections were washed again three times with PBS and mounted using Vectashield® mounting medium (containing 1.5 µg/ml DAPI). Pictures were taken using the inverted microscope DMI6000B (Leica) as well as the fluorescence microscope BZ-9000 (Keyence). Staining was quantified using the BZ-II Analyzer software (Keyence).

3.4.3) Whole mount zebrafish immunohistochemistry

Zebrafish embryos were de-chorionated manually and then fixed in 4% Paraformaldehyde overnight at 4 °C. The embryos were washed with 100% methanol and then stored at -20 °C for long term. On the day of immunostaining the embryos were taken out from 100% Methanol and rehydrated as following

75% Methanol + 25% PBST – for 5 min at RT

50% Methanol + 50% PBST – for 5 min at RT

25% Methanol + 75% PBST – for 5 min at RT

The embryos were then incubated with ddH₂O for 1 hour on a moving platform, in eppendorf tube kept sideways. After 1 hour, the embryos were placed in 100% acetone (Chilled) to facilitate permeabilization, for 20 min. The embryos were then rinsed two times with PBST (1% triton in PBS) for 5 min. After washing the embryos were treated with collagenase (1mg/ml). Depending upon the age of zebrafish the embryos were treated for different time intervals. For embryos aged between 0-24 hours the treatment was skipped. For Day1- Day3 embryos the treatment was done for 45 min at RT and for fish older than 3 days treatment was done for 60 min. After the treatment the embryos were washed two times again with PBST for 5 min. The embryos were then blocked in 10% BSA in PBST. Blocking was done overnight with gentle swirling at 4 °C. Next day the embryos were stained with primary antibody

(1:200) and incubated overnight at 4 °C with gentle swirling again. Next day, the embryos were washed for 3 hours with PBST with gentle shaking. The secondary antibody diluted in PBST was added and embryos were kept overnight for staining. After 24 hours the embryos were washed 2 times with PBST and transferred to 80% glycerol and 20% ddH₂O for imaging.

3.4.4) Whole Mount in situ hybridization

Zebrafish embryos, about 10 from each stage of development, were fixed in 4% Paraformaldehyde overnight at 4°C, and then washed several times with PBS. Fixed samples were rinsed with PBST (0.2% Tween 20, 1.4 mM NaCl, 0.2 mM KCl, 0.1 mM Na₂HPO₄, and 0.002 mM KH₂PO₄; pH 7.4), treated with 10 µg/ml proteinase K for 10 min, washed with PBST several times, and then fixed again in 4% PFA in PBS for 20 min. After brief washing with PBST, the embryos were incubated with hybridization buffer (HyB) containing 50% formamide, 5x SSC (For 20x SSC stock solution: NaCl 175.3 g, Citric acid trisodium salt 88.2g dissolved in 1l of water) , and 0.1% Tween 20 for 5 min at 65°C. Pre-hybridization was performed in HyB+, which is the hybridization buffer supplemented with 500 µg/ml yeast tRNA and 50 µg heparin (Sigma) for 5 hours at 65 °C. After pre-hybridization, samples were incubated in 100 ng of the RNA probe in 200 µl of HyB+ at 65 °C overnight for hybridization. Embryos were then washed at 65 °C as follows;

1. Hyb buffer, at 67 °C, 20 min (1x)
2. 50% SSCT/50% Formamide, at 67 °C, 3x 20 min
3. 75% SSCT/25% Formamide, at 67 °C, 1x 20 min
4. SSCT at 67 °C, 2x 20 min
5. SSCT (0.2x) at 67 °C 4x 30 min
6. PBT, 67 °C, 5 min

After serial washings, embryos were incubated in blocking solution containing 5% sheep serum and 10 mg/ml BSA in PBST for 2 hours and then incubated in 1:2000 alkaline

phosphatase conjugated anti-dig antibody in blocking solution over night at 4 °C. After the reaction, samples were washed with PBST several times and transferred to the staining buffer. The staining reaction was held with NBT (50 mg Nitro Blue Tetrazolium dissolved in 0.7 ml of N,N-dimethylformamide anhydrous and 0.3 ml of water (store at -20°C)) : BCIP (50 mg of 5-Bromo 4-Chloro 3-indolyl Phosphate dissolved in 1 ml of N,N-dimethylformamide anhydrous (store at -20°C)), (1:5) in staining buffer until the signal was strong and easily visible. Samples were then observed and documented with a binocular microscope (Leica).

Hybridization buffer	For 50 ml
50 % formamide	25 ml
5 x SSC	12.5 ml
50 µg/ml heparin	50 µl of 50 mg/ml
500 µg/ml tRNA	500 µl of 50 mg/ml
0.1 % Tween 20	250 µl of 20 %
Sterile H2O	to 50 ml
0.092 M citric acid	460 µl of 1 M
(To adjust pH to 6.0)	

3.5) DASPEI live staining

Zebrafish embryos were immersed in 1mM DASPEI (2-(4-dimethyl-aminostyryl)-N-ethyl pyridinium iodide) in E3 (5mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄) for 30 min according to Balak et al. (Balak et al., 1990). They were then rinsed thoroughly in E3, anaesthetized with Tricane (1X) and were observed under the fluorescence microscope.

3.6) Fluorescence-activated cell sorting (FACS)

For cytometric analysis 10^7 – 10^8 cells were harvested with TrypLE-express, washed with PBS, and subsequently re-suspended in 100 μ l of PBS. Probes were sorted by flow cytometry on FACS Aria2 (BD Biosciences). Data were analysed with the FlowJo software (Tree Star Inc., OR, USA). GFP-positive and GFP-negative cells were either plated to 6-well plates and GFP fluorescence was monitored over 5 or more depending upon the experiment, in culture or were processed directly for real time PCR analysis. Cells were examined under a fluorescent microscope before plating to exclude the presence of green cells in the GFP-negative population. The rest of sorted cells were used for RNA preparation. For RT and real time PCR analysis RNAs were extracted by RNA mini kit (Qiagen). Residual genomic DNA was removed by DNase I treatment (Qiagen). RNA was reverse transcribed using random hexamers and Moloney murine leukemia virus reverse transcriptase (M-MLV, Invitrogen).

3.7) Animals

3.7.1) Zebrafish

Different strains of zebrafish were kept in fish water at 28.6 °C and fed well under 14:10 light dark photoperiod, controlled by automatic timer in controlled conditions (Westerfield 2000; the zebrafish book, ZFIN). Zebrafish strains selected to breed were kept in fish tanks and were allowed to mate. Eggs were collected 10 min after and were noted as hpf 0 (hours post fertilization). The embryos were transferred to fish water with 0.01 % PTU (1-Phenyl-2-thiourea) to prevent them from pigmentation. The embryos were de-chorionated manually using needles at 24 hour after hpf 0. The de-chorionated embryos were then transferred to fresh fish water with 0.01% PTU in new Petri dish and kept in an incubator at 28.6 °C until collected at the desired day of development.

3.7.2) Morpholino microinjection

Microinjection into zebrafish embryos is a well-established and robust technique for exploring the role of a particular gene in development. Micropipettes were fabricated by heating and

pulling borosilicate glass capillary tubes (World Precision Instruments, Inc., 1B100-4) in a micropipette puller device (Sutter Instruments Inc., Flaming/Brown P-97). 1.5% agarose (American Bioanalytical, Inc., AB00972-00500) in 1x E3 medium plates moulded with wedge-shaped troughs were poured into the petri dish which served as an easy method for holding the embryos during the injection. The embryos were manipulated so that the cytoplasm of the 1-cell stage embryo is visible under the dissecting microscope. The chorion and then the yolk sac were penetrated with the micropipette in order to inject into the embryo.

4. RESULTS

4.1) Identification of genes essential for serotonergic specification

4.1.1) Evaluation of serotonergic *in vitro* differentiation protocol

In this part of the study we used an embryoid body (EB) protocol as a basis for the evaluation of serotonergic *in vitro* differentiation of mouse ES cells.

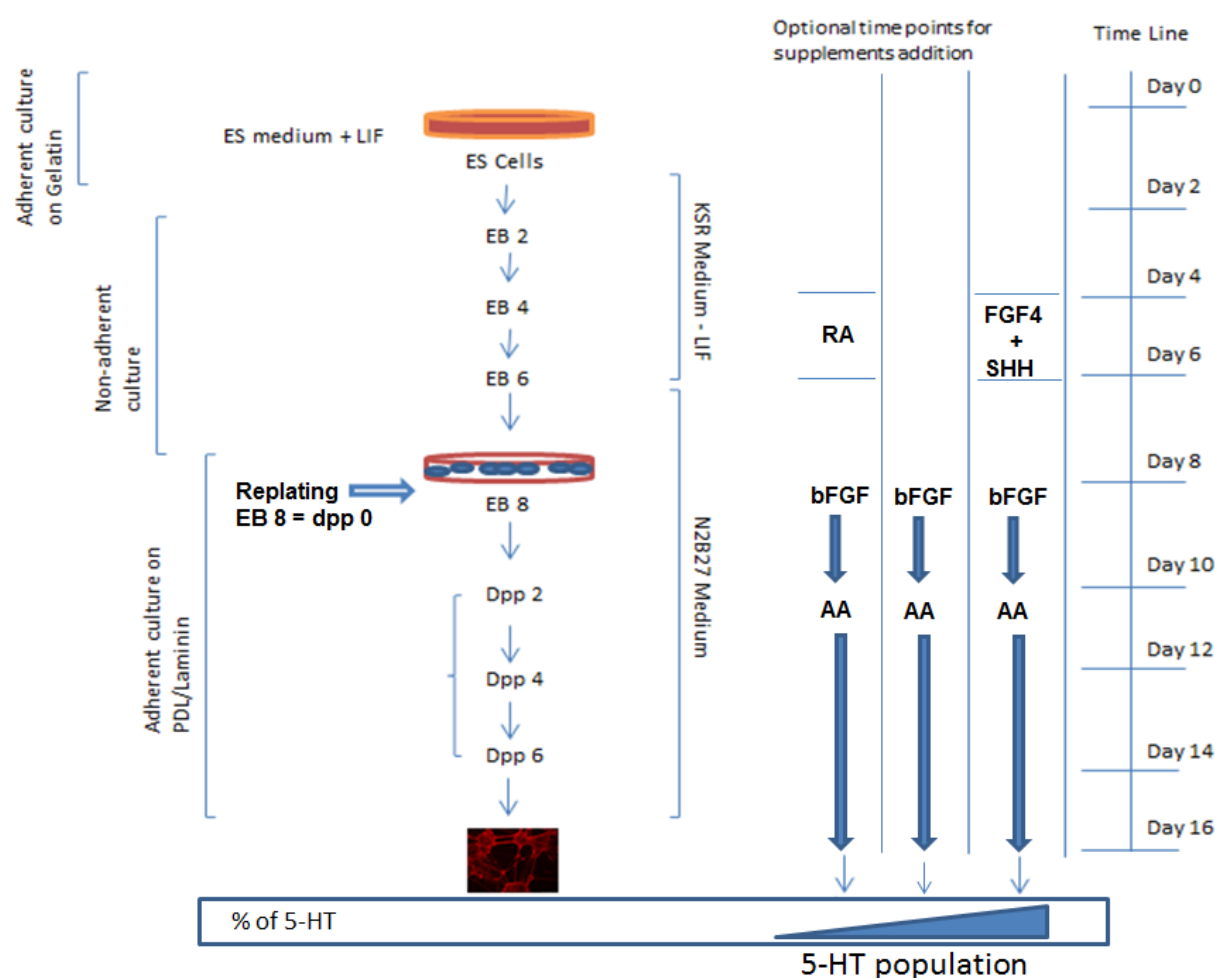


Figure: 12. EB protocol for the differentiation of embryonic stem cells into serotonergic neurons. EBs, embryoid bodies; LIF, Leukemia inhibitory factor; KSR, Knockout serum replacement; SHH, sonic hedgehog; FGF, fibroblast growth factor; BDNF, brain derived neurotrophic factor; PDL, poly-D-lysine; Lam, laminin; AA, Ascorbic acid; RA, Retinoic acid; N2-medium, DMEM/Nutrient mixture F12 medium supplemented with N2 additives; N2B27, mixture 1:1 N2 medium and B27 medium (Neurobasal medium supplemented with B27 additive).

The protocol previously established in our lab (Alenina et al., 2006) includes, first cultivation of mouse ES cells in a serum and LIF containing medium (Figure: 12). The differentiation is

initiated by formation of EBs in non-adherent plastic dishes under serum-free conditions (KSR medium) and LIF withdrawal. EBs are like floating balls of cells, which are formed by ES cells after their re-plating on non-adherent surface and LIF withdrawal. They contain the precursors of all three germ layer derivatives. EBs were cultured for 6 days in KSR medium and 2 more days in neuronal-differentiation medium N2B27. After 8 days, these EBs were dissociated and plated in N2B27 medium onto PDL/Laminin coated dishes, a substrate which facilitates the attachment of neuronal precursor cells. After re-plating neural precursor cells were first enriched for 2 days by the addition of bFGF, which is a strong mitogen and then terminal differentiation of neurons was completed after withdrawal of this growth factor. The culture was further maintained in N2B27 medium with addition of ascorbic acid (AA) for the better survival of neuronal cells. The appearance of neurons and serotonergic neurons was followed after re-plating on PDL/Laminin matrix by immunocytochemical analysis for the pan-neuronal marker, *Tuj1*, and the serotonergic marker, 5-HT, respectively. After Dpp7 the cells usually start dying, mainly because of contact inhibition and increased density of cells. However, 5-HT positive cells were observed from Dpp3 and in abundance from Dpp7 onwards (Figure: 13). One of the advantages of this protocol was that it not only gives a relatively high yield of serotonergic neurons, but also does not need feeder cells, like PA6 (Kawasaki et al., 2002).

Retinoic acid (RA) is a classical agent which is usually used for the induction of neuronal differentiation *in vitro*. Treatment with RA at the EB-stage of differentiation leads to the generation of a virtually pure population of neuronal precursors which are able to generate a remarkably homogeneous population of neurons *in vitro* that form functional synaptic connections (Bibel et al., 2004). To check if RA treatment may improve the outcome of differentiation we applied RA at the EB4 stage for 2 days of differentiation (4-/2+/2- format). Whereas this protocol produced a high number of Tuj1-positive neurons, the number of 5-HT-

positive cells went dramatically down, indicating that RA induction does not favour the differentiation toward serotonergic phenotype, confirming previous studies of our group.

We also evaluated if application of FGF4 and SHH – two factors which are essential for the formation of 5-HT neurons *in vivo* (Chung et al., 2002) and *in vitro* (Ye et al., 1998) may influence the rate of serotonergic cell formation in our protocol. Although a slight improvement in serotonergic neuron production was detected in some experiments, no drastic increase in the number of 5-HT-positive cells was observed upon application of these agents.

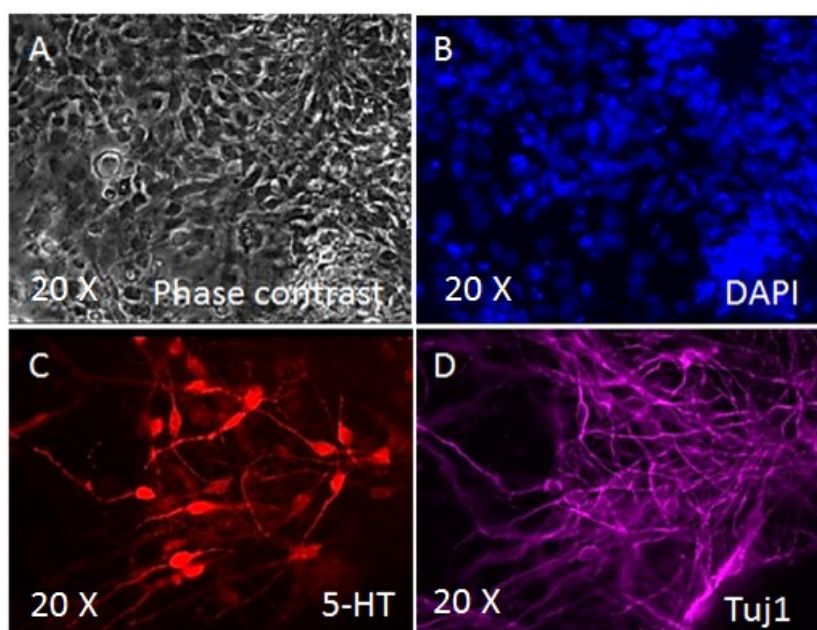


Figure: 13. Serotonergic differentiation of mouse E14Tg2a ES cells. Double immunofluorescence analysis of Tuj1 (violet)- and 5-HT (red)-positive neurons, performed at differentiation day 20. Nuclei are labeled with DAPI.

4.1.2) Comparative analysis of microarray datasets of *in vivo* and *in vitro* serotonergic differentiations

In order to find the genes that might be involved in the process of differentiation and development of serotonin producing neurons, the microarray data (transcriptome analysis) of three independent experiments were analysed. Two data sets were obtained from *in vitro* differentiation of ES cells towards serotonergic phenotype which revealed subsets of genes differentially regulated along the process of differentiation (microarray 1 and 2). The third

data set compared the transcriptome of serotonergic versus non-serotonergic neurons obtained from mouse brain.

Microarray 1: Functional Genomics in Embryonic Stem cells (FunGenES) was an Integrated Project funded by 6th framework program of the European Union that aimed to achieve a basic understanding of the process of self-renewal and differentiation (Schulz and Kolbe et al., 2009). The idea of this integrated consortium was to develop an efficient protocol for the differentiation of ES cells into lineage committed cells of all the three germ layers. This controlled differentiation protocol intended to provide a selectable system for each cell type that will be suitable for affymetrix analysis, and hence discovery of unknown candidates for further functional screening. One of the partners of this consortium (Sanofi Aventis Pharma R&D, Vitry Research Center, France) used the E14Tg2a ES cell line for the comparison of transcriptomes of cell populations obtained in the course of *in vitro* differentiation (EB protocol) with or without RA induction (4-/2+/2- format). They performed successful neuronal differentiation and collected RNA at different time points (EB0, EB4, EB6, EB8, dpp2, dpp5) of RA-treated (4-/2+/2- format) and control differentiations and used these samples for microarray analysis. Cluster analysis revealed the existence of a group of genes (Cluster 1) whose expression was up regulated at day 13 of differentiation in RA-free conditions (Figure: 14). This cluster included all the key genes like *Tph2*, *VMAT*, *FEV*, and *SERT*, which are markers of serotonergic neurons. We hypothesized that this cluster may also contain other yet unknown genes which play a role in serotonergic specification.

Microarray 2: During previous work of our lab the *in vitro* differentiation protocol (4.1.1) was applied to *Lmx1b* overexpressing cells derived from the CGR8 ES cell line (Dolmazon et al., 2011). *Lmx1b* is a transcription factor, essential for the serotonergic specification *in vivo* (Cheng et al., 2003) and its overexpression was shown to promote the formation of 5-HT neurons in *in vitro* studies (Figure: 8, Dolmazon et al., 2011). RNA from *Lmx1b* overexpressing cells (clone *Lmx1b*-L1) and of the parental CGR8 line were collected at

different time points of differentiation (EB0, EB6, EB8, dpp4, and dpp8) and subjected to affymetrix analysis. Upon cluster analysis and gene expression profiling we found out that a relatively large cluster is enriched in the Lmx1b-L1 with all the characteristic 5-HT genes like *Tph2*, *SERT*, *VMAT* and *Pet1* (Figure: 14).

Microarray 3: Finally, we looked into already published data sets. Here, in order to understand the molecular architecture of developing serotonin neurons, the group of Evan Deneris purified post-mitotic embryonic 5-HT neurons by flow cytometry. They identified significantly enriched expression of hundreds of unique genes in 5-HT neurons, probably also containing new serotonergic markers (Wylie et al., 2009). Two distinct sample clusters were identified in the work corresponding to the serotonergic and non-serotonergic cell groups. Interestingly, the six serotonergic profiles sorted into two sub-clusters that correspond to the rostro-caudal origins of serotonin neurons, provided the first clear indication of large gene expression differences in rostral and caudal serotonin neurons. It was also evident that rostral and caudal serotonin neurons were more similar to each other than to non-serotonergic neurons from the same rostro-caudal level (Wylie et al., 2009). We compared our data with the unique transcript enriched in both rostral and caudal 5-HT neurons since a major aim of this work was to identify new potential transcriptional determinants of 5-HT neuron.

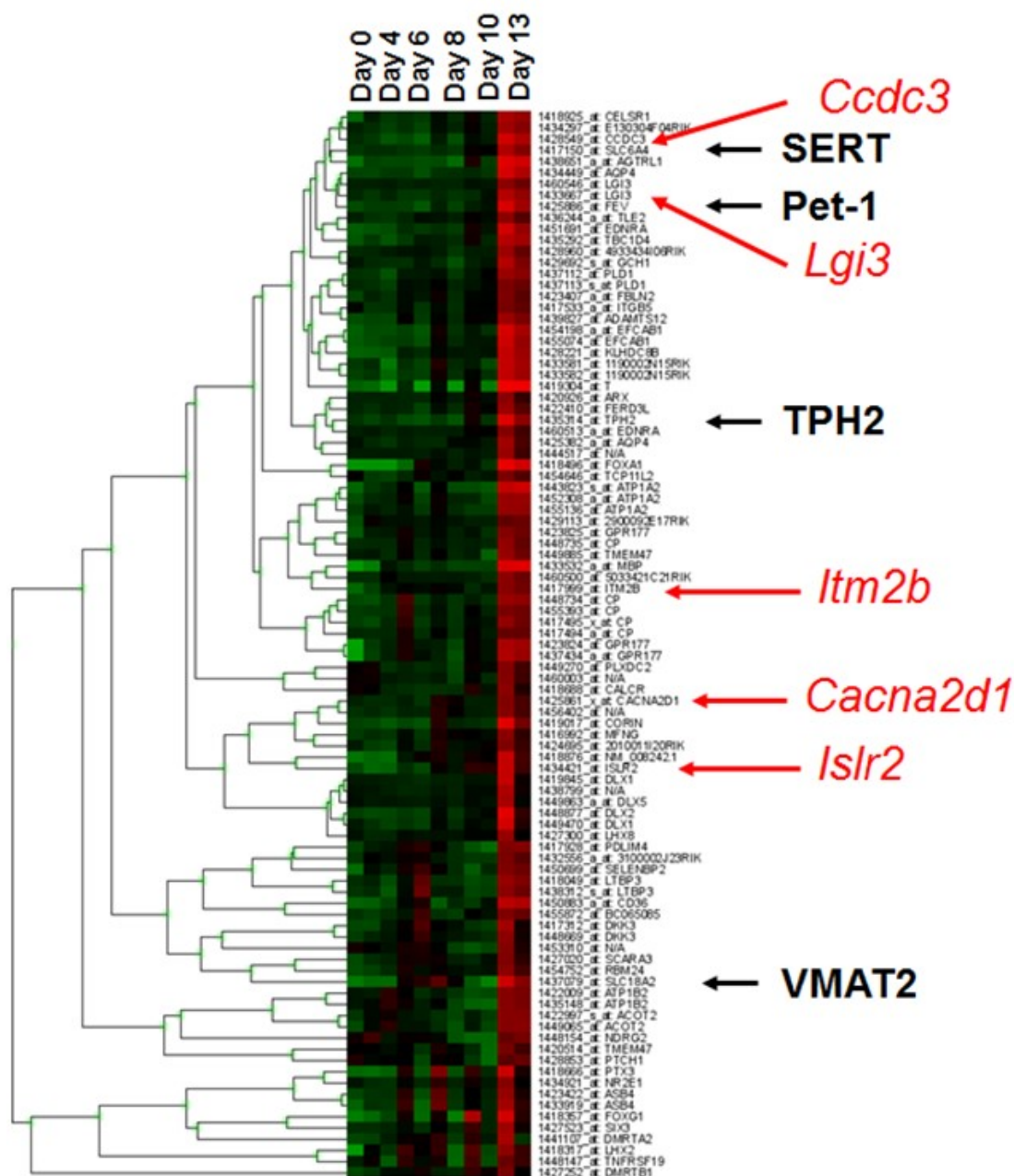


Figure: 14: Heat map defining the “*Tph2* Cluster” during days of differentiation with selected candidate genes (Microarray 1). Selected candidates *Ccdc3*, *Lgi3*, *Itm2b*, *Cacna2d1* and *Islr2* (red arrows), were present in the same cluster which contained other serotonergic marker genes like *TPH2*, *VMAT2*, *Pet1* and *SERT* (black arrows) (<http://www.bioinf.ebc.ee/fungenes/Clusterings?AVEF.1/pict/1.html>, Pradier et al., unpublished).

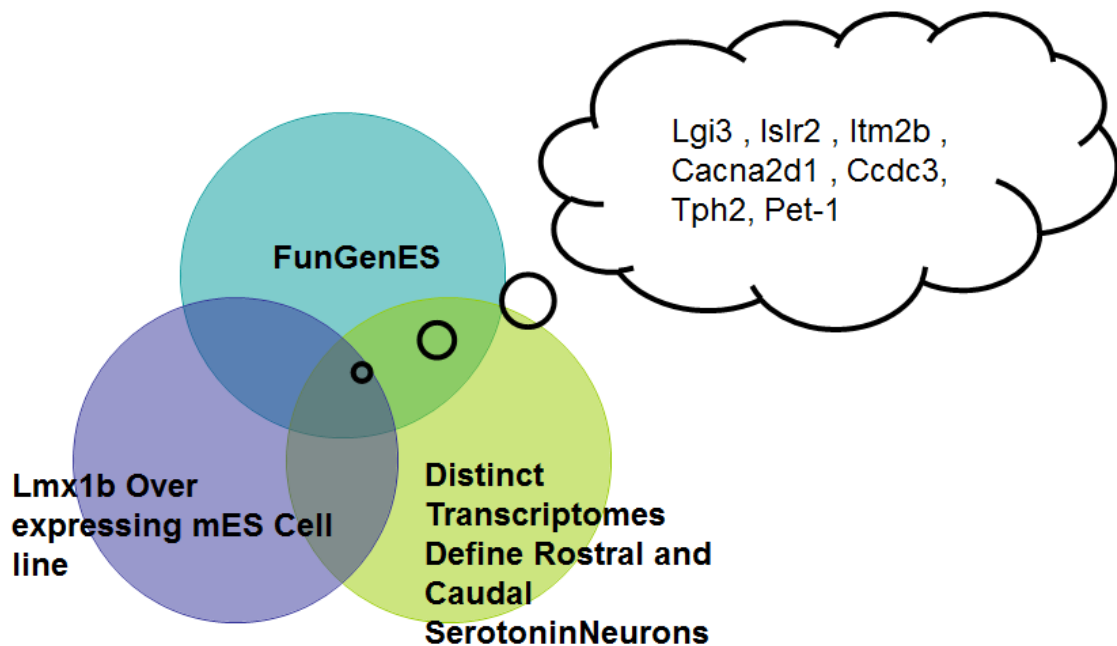


Figure: 15. 5 genes which were common in all the three microarray datasets; *Lgi3*, *Ccdc3*, *Cacna2d1*, *Islr2* and *Itm2b*.

Upon comparing, the repertoire of gene sets from three independently carried out experiments; we found 5 genes which were common in all the three microarray datasets. They were picked as the prime candidates for further analysis. These genes were *Lgi3*, *Ccdc3*, *Cacna2d1*, *Islr2* and *Itm2b* (Figures: 14 and 15). Also, the other important serotonergic defining genes like *Tph2* and *Pet-1* were found to be overlapping along with this group of new candidates.

4.2) Validation of candidate genes

The expression of these candidate genes was validated at transcriptional level. In this part of the study, we applied the differentiation protocol to 3 different lines – mouse ES cells, and rat

and mouse *iPS* cells to validate the robustness of candidate gene expression during serotonergic *in vitro* differentiation. Further on, we evaluated the expression of these genes in mouse brains analysing the *in situ* hybridization data available from the Allen Brain Atlas.

4.2.1) Mouse ES cells

To validate the candidate gene expression in mouse ES cells we took *Lmx1b*-overexpressing cells, which results in enriched population of serotonin-positive cells at the end of the differentiation in comparison to the parental CGR8 line (Figure: 8). The relative expression of 4 candidate genes (*Lgi3*, *Cacna2d1*, *Itm2b* and *Islr2*) was evaluated along the course of differentiation by real time PCR (Figure: 16). The expression of *Lgi3*, *Cacna2d1*, and *Islr2* showed a similar pattern as it was observed in the affymetrix screen, being low in ES cells and at the early stages of differentiation, and being drastically increased at the late stages of neuronal differentiation. Moreover, the high expression level of *Lgi3* and *Cacna2d1* was observed only in serotonin-enriched *Lmx1b*-overexpressing cells, but not in the parental CGR8 line (Figure: 16A and 16B), whereas *Islr2* expression was up regulated in both, *Lmx1b* and CGR8 cells (Figure: 16D). The expression of *Itm2b* was found to be discordant to the microarray data (Figure: 16C). Due to methodological problems we were not able to analyse the expression of *Ccdc3* in this experiment.

Thus, this analysis revealed that *Cacna2d1* and *Lgi3* are the most promising candidates for further studies.

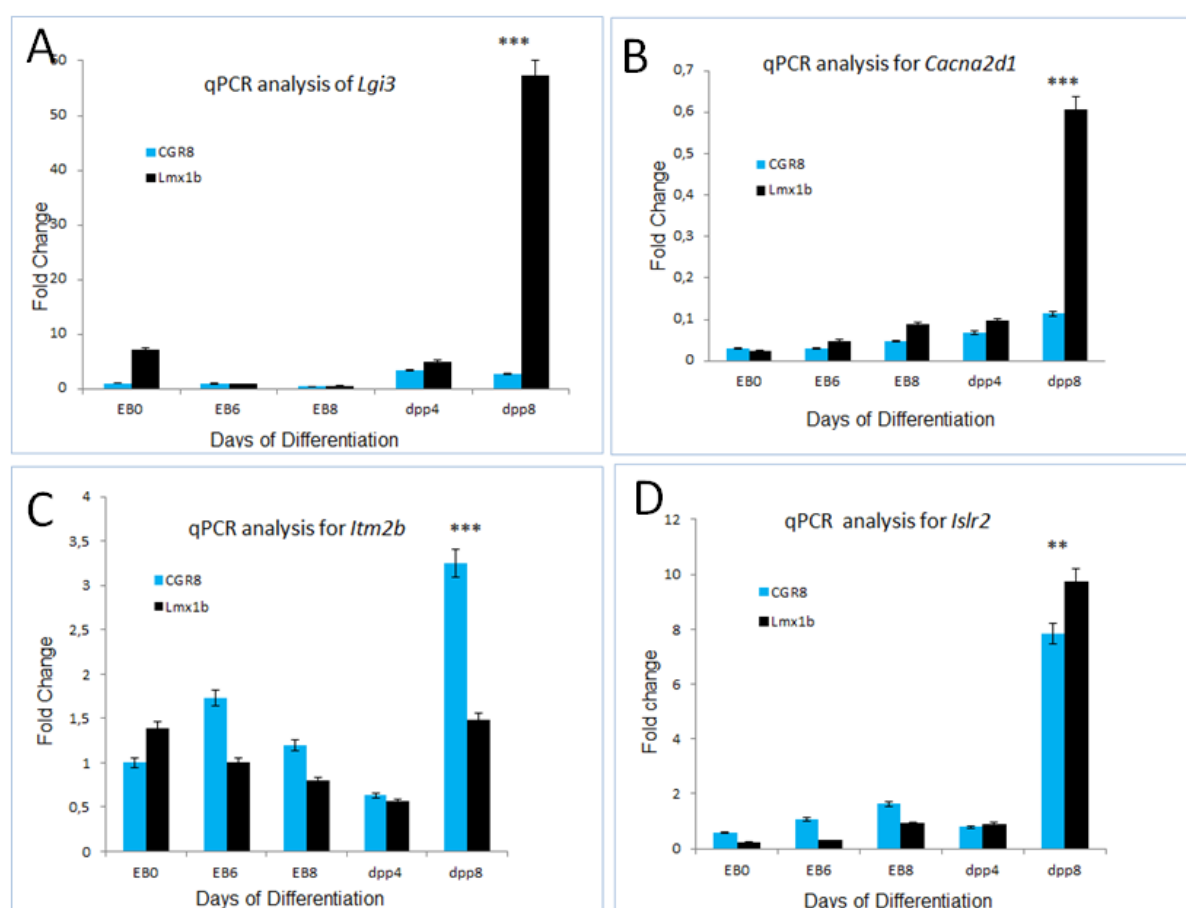


Figure 16. qPCR analysis of *Lgi3* (A), *Cacna2d1* (B), *Itm2b* (C), and *Islr2* (D) expression during the course of differentiation in Lmx1b overexpressing and CGR8 parental lines. EB – embryoid bodies. Dpp – days post plating. EB0, EB6, EB8, dpp4, dpp8 – day0, 6, 8, 12, and 16 of differentiation, respectively. The expression of *Lgi3* and *Cacna2d1* was higher in Lmx1b line than in CGR8 at the final stage of neuronal differentiation. *** $P < 0.00001$, ** $P < 0.001$.

4.2.2) Rat iPS cells

Derivation of rat iPS cells

To derive rat iPS (riPS) cells, rat embryonic fibroblasts (REFs) were transduced with *Oct4*, *Sox2*, *Klf4*, *cMyc*, and for visualization, with *EGFP*-encoding lentiviruses (OKSM+G). They were plated at low density on mitomycin-inactivated mouse embryonic fibroblasts (MEF) feeder layer in media permissive for the propagation of *iPS* cells. In the first set of experiments the original cell culture conditions, which was described for mouse *iPS* cell, derivation (Yamanaka et al., 2006) using serum- and LIF-containing medium was used. However, in contrast to previously reported success in riPS cell derivation using similar

transduction and cell culture systems (Liao et al., 2009) we were not able to get any viable *riPS* cell colonies (Figure: 17).

Reprogramming Medium	Reprogramming efficiency	Total number of picked clones	Total number of survived clones	Survival rate after 2 passage
DMEM + 15 % FCS + LIF	0	0		
DMEM + SR + 2i + LIF + A-88-01	$8.9 \pm 1.3 \times 10^{-4}$	48	37	$25 \pm 12.5 \%$
N2B27 + 2i + LIF	$5.4 \pm 0.9 \times 10^{-4}$	48	37	$70.8 \pm 6.3 \%$

Figure: 17. Efficiencies of rat iPS cell derivation in different culture conditions. To derive rat iPS (rips) cells 1.5×10^5 REFs were transduced with the LVTHM-based Oct4, Sox2, Klf4, cMyc and EGFP lentiviruses. Four days later cells were split onto a 10 cm culture dish and cultured in one of the three reprogramming media. After 10-12 days, primary riPS cell colonies were counted to calculate the reprogramming efficiency. Survival rate was calculated as a relation between the number of picked and the number of surviving clones after passage 2. $P < 0.05$, Students's t-test conditions 3 vs condition 2, [DMEM + SR + 2i + LIF + A-83-01] N = 3; [N2B27 + 2i + LIF]: N = 4 independent experiments.

The N2B27+2i+LIF culture condition was then applied and a robust derivation of riPS cells from REFs with the help of (OKSM+G) lentiviral vectors was achieved (Data not shown).

The N2B27+2i+LIF medium was then compared with previously reported most efficient rat iPS cell-derivation medium containing serum replacement (KSR), MEK and GSK3 inhibitors (2i), LIF, and additionally, the activin receptor-like kinase (ALK5) inhibitor A-83-01 (Li et al., 2009). The numbers of primary rat iPS cell colonies were slightly higher in the KSR-based medium; however, the percentage of surviving clones following 2 subsequent passages was 71% in the N2B27-based vs. 25% in the KSR-based medium, respectively.

Eight clones reprogrammed in N2B27+2i+LIF conditions were picked and propagated to establish rat iPS cell lines. The cells were routinely maintained on inactivated MEFs in N2B27+2i+LIF medium. Rat iPS cells typically grew as compact clumps that tend to detach from substrate as colonies expanded, resembling in this respect rat ES cells. However, after

several rounds of trypsinization and passaging, riPS cells changed their morphology, and were more flat and adhesive. Also noticeable, primary rat iPS cell colonies were frequently surrounded by small satellite colonies (Figure: 18, central panel).

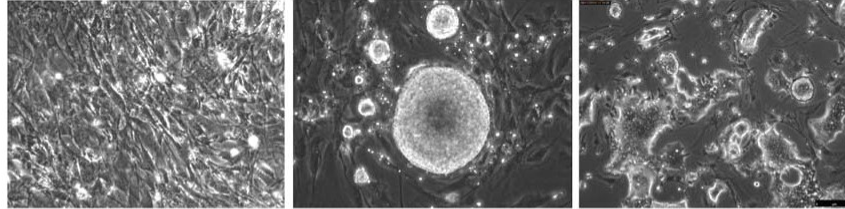


Figure: 18. Generation of rat iPS cells. Rat embryonic fibroblasts (REFs) (left), primary riPS cell colony derived thereof after transduction with lentiviruses expressing *Oct4*, *Klf4*, *Sox2*, *cMyc*, and *EGFP* with some satellite colonies (middle), and rat iPS cell line after several passages in N2B27+LIF+2i medium (right).

A clonability assay was then performed which shows that riPS cell proliferation and survival were severely compromised in the absence of the GSK3 inhibitor CHIR99021, whereas omission of PD0325901 and LIF from the culture medium led to the quick differentiation of riPS cells (Figure: 19).

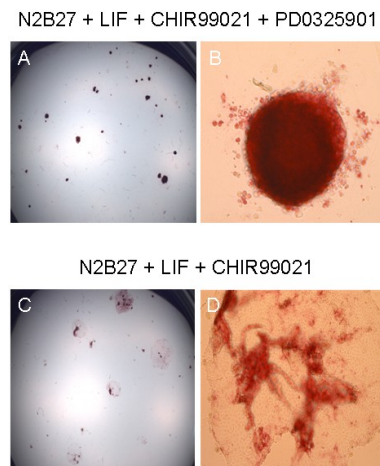


Figure: 19. RiPS cell clonability assay. Representative images of alkaline phosphatase staining of riPS cells, seeded at a density of 500 cells per well of a 6-well plate and cultured for 5 days in the presence of LIF and 2i (A, B), or in the presence of LIF and CHIR99021 (C, D). 2.5x magnification (A, C). Examples of an undifferentiated colony (B) and of a morphologically differentiated colony with weak AP-staining (D) (20x and 10x magnification, respectively). Similar results were obtained in 2 independent experiments.

Pluripotency of rat iPS

Immunostaining for pluripotency markers revealed that *riPS* cells were positive for Oct4, Nanog, SSEA1 and alkaline phosphatase (Figure: 20A and Figure: 20A–C). Also, the *riPS* cell sub-clones were able to differentiate *in vitro* by formation of embryoid bodies (Figure: 20B, C).

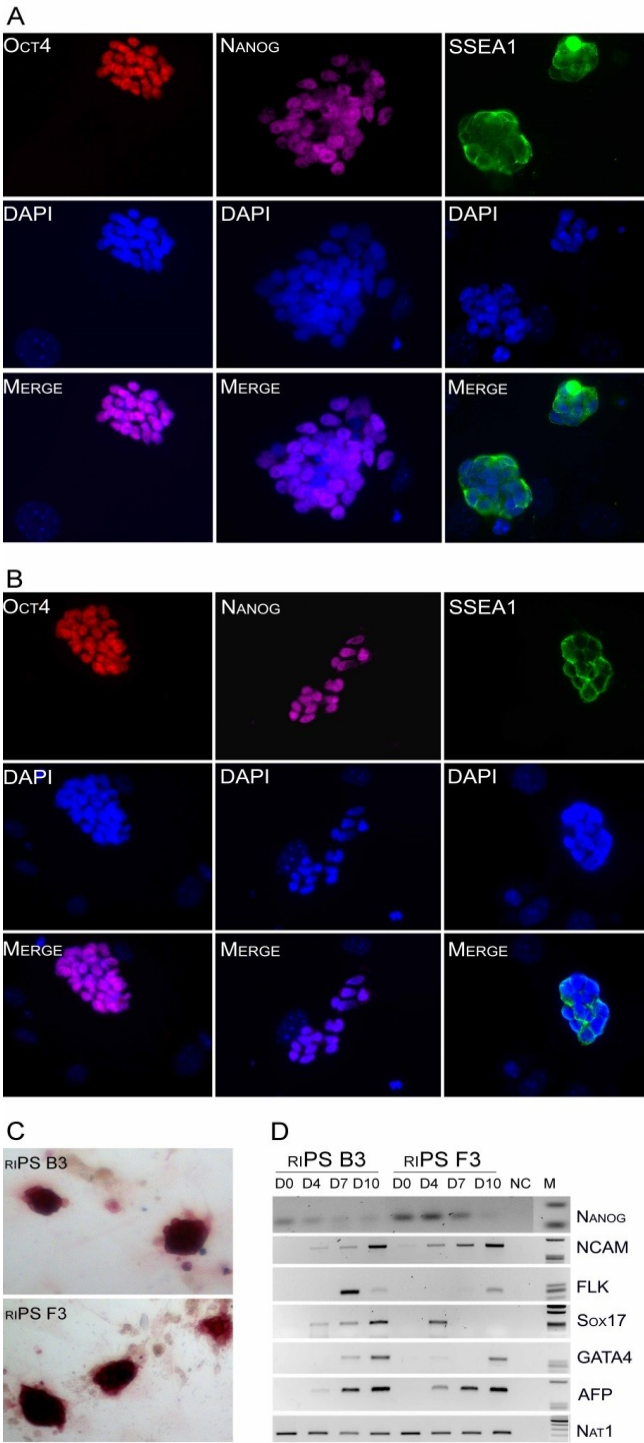


Figure: 20. Expression of the pluripotency markers Oct4, Nanog, and SSEA-1 in the primary riPS cell clones IVB3 (A) and IVF3 (B) (passage 13), shown by immunocytochemical staining with respective antibodies. (C) Alkaline phosphatase staining in the same two clones. (D) RT-PCR analysis of pluripotency (Nanog), ectoderm (NCAM), mesoderm (FLK and AFP), and endoderm (Sox17 and GATA4) lineage marker expression during the time course of differentiation of the same riPS cell clones using embryoid body (EB) protocols. Cells were harvested at the indicated days after the EB formation (D0-D10). Nat1 served as an endogenous mRNA control. NC- negative control.

A high rate of EGFP-encoding lentivirus silencing was observed in newly derived riPS cell clones, which became more obvious with increasing number of passages (Figure: 21A–C). Interestingly, while the silencing appeared in undifferentiated cells, reactivation of lentiviral expression was observed in some spontaneously differentiated cells (Figure: 21D, E).

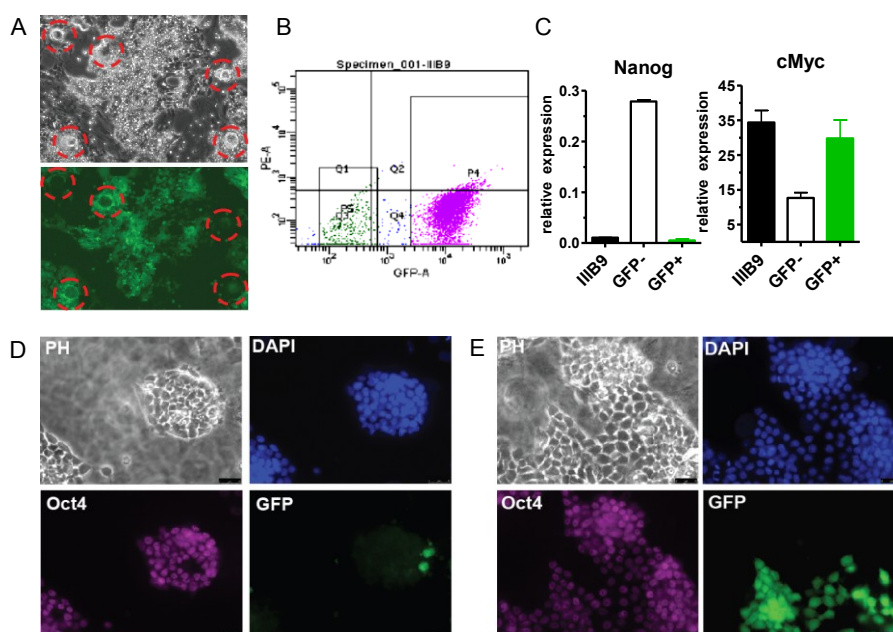


Figure: 21. Lentivirus silencing in undifferentiated rat iPS cells. (A) riPS cell clone IIIB9 after 22 passages (p22) in culture. (B) FACS for EGFP⁺ cells of rat iPS cell clone IIIB9 p22. (C) Real time PCR analysis of sorted cells, showing that EGFP⁻ rat iPS cells express high levels of Nanog, confirming the pluripotent state of this cell population. IIIB9: N=3; EGFP⁻, EGFP⁺: N=2. (D) The EGFP⁻ fraction of rat iPS cells after FACS shows the onset of EGFP expression in some cells already after 1 day in culture. (E) EGFP⁻ fraction cultured for further 4 days shows a substantial number of EGFP⁺ cells, which exhibit the morphology of differentiated cells.

Neuronal differentiation of rat iPS cells

In order to produce a population of serotonergic neurons for the evaluation of candidate genes we applied the protocol developed in mouse ES cells (4.1.1) to rat iPS cells. Using this method we could generate neurons (Tuj1-positive cells, Figure 22). However, no 5-HT-

positive cells appeared in the culture. Therefore, we did not perform the analysis of candidate genes in these experiments and just analysed the expression of general differentiation markers and the neuron-specific marker, Nestin, by RT-PCR during the course of differentiation (Figure 22 A).

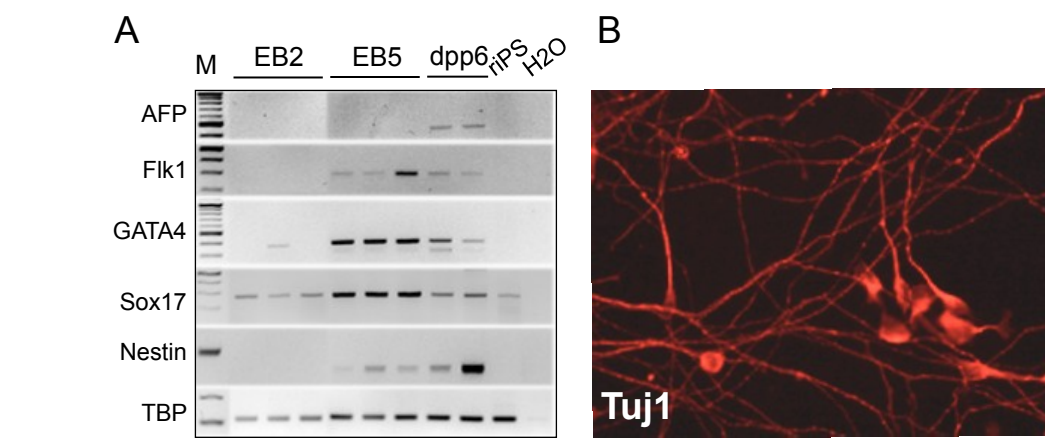


Figure: 22. Neuronal differentiation of rat iPS cells. A. RT-PCR analysis of gene expression in undifferentiated rat iPS cells (clone H5) and in cells during the course of *in vitro* differentiation via the embryoid body (EB) protocol after 2 days (EB2), 6 days (EB6) and 12 days (dpp6, 6 days post plating) of differentiation; shown is the analysis of mesodermal (*AFP*, *Flk1*), endodermal (*Gata4* and *Sox17*), and ectodermal marker (*Nestin*) expression. (B) Differentiation of rat iPS cells into neurons *in vitro*, shown by immunostaining with Tuj1 antibodies at dpp6 of differentiation (clone H5).

4.2.3) Mouse iPS cell

Derivation of mouse iPS cells

For the evaluation of candidate genes we generated mouse iPS cells from a *Tph2::eGFP*^(FRT-neo-FRT) mouse with targeted EGFP insertion into the *Tph2* locus (Migliarini et al., 2013). The serotonergic neurons of this line exhibit GFP fluorescence *in vivo* in the brain and we hypothesized that iPS cells derived from this mouse line will be instrumental in separation the serotonergic *versus* non-serotonergic cells in *in vitro* studies, thus enabling the analysis of a pure serotonergic population obtained in course of *in vitro* differentiation.

To generate mouse iPS cells the methodology established during derivation of rat iPS cells was applied to mouse. For mouse iPS cell generation, MEFs were isolated from E13 embryos of a *Tph2::eGFP*^(FRT-neo-FRT) mouse and transfected with a single polycistronic pseudolentiviral vector carrying *Oct4*, *Sox2*, *cMyc* and *Klf4* genes driven by a doxycycline (DOX)-inducible

promoter (Craey et al., 2009). After DOX-induction 25 primary colonies of reprogrammed cells were picked (with the effectiveness of reprogramming, 2×10^{-4}) and propagated in *miPS* medium. After several passages 4 clones (404, 405, 403, 402) were selected by their stem-cell like morphology and amplification ability for further analysis. To check whether they possess characteristics of stem cells, we evaluated the expression of pluripotency markers by immunofluorescence analysis. Clone 404 showed clear reactivation of Nanog and Oct4 (Figure: 23), confirming successful reprogramming to the pluripotent state and could keep its stem-like characteristics at least for 10 passages in the absence of DOX. These cells were primary cultured on feeder layer, but were later adopted to feeder-free conditions and maintained on gelatine, that did not affect the stem cell-like morphology, proliferation and expression of pluripotency markers in these cells. This line was further used for the differentiation into serotonergic neurons.

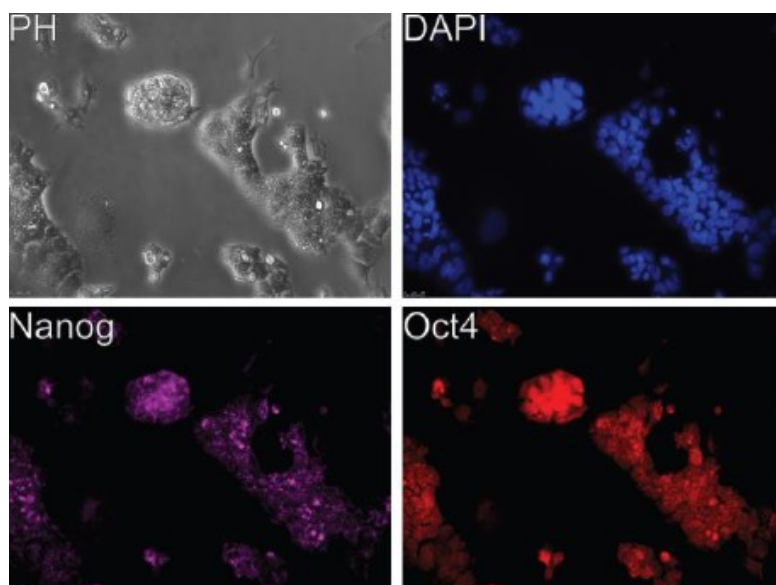


Figure: 23. Immunocytochemical analysis of mouse iPS cell clone 404 (passage 10) for Oct 4 (red) and Nanog (violet) expression. PH –phase contrast. DAPI: nuclei are shown in blue.

Differentiation of *miPS* to serotonergic neurons and evaluation of candidate genes

To differentiate *Tph2::eGFP*^(FRT-neo-FRT) *miPS* to serotonergic neurons we used an EB protocol established for ES cell differentiation (4.1.1). GFP-positive cells were observed from Dpp5 and in abundance from Dpp8 onwards (Figure: 24). Co-staining for the pan-neuronal marker

Tuj1 showed that only part of neurons obtained in the course of the differentiation were GFP-positive. In contrast, GFP and 5-HT staining showed a 100 percent co-localization confirming that GFP is a reliable marker of serotonergic neurons in iPS404 cells.

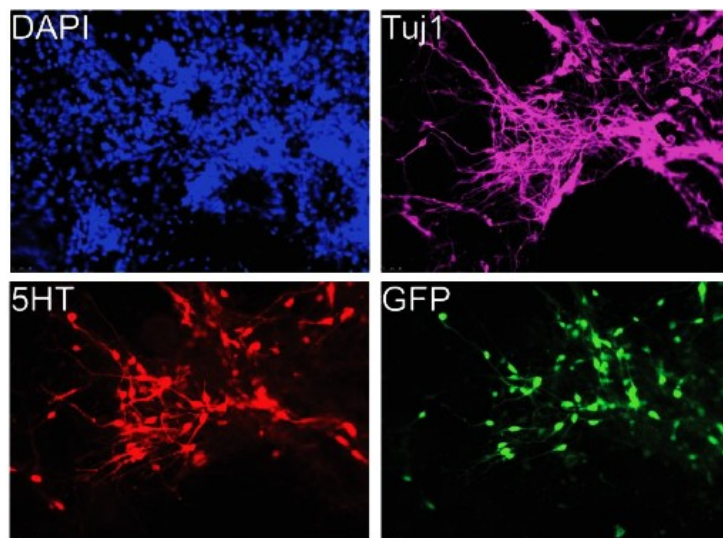


Figure: 24. Differentiation of *Tph₂::eGFP^(FRT-neo-FRT)* miPS404 into serotonergic neurons. Immunocytochemical analysis of miPS404 cells at dpp14 of differentiation for neuronal marker Tuj1 (violet), serotonin (red) and life EGFP fluorescence (green).

To evaluate candidate genes during the course of mouse iPS404 cell differentiation, we separated GFP-positive and GFP-negative cells by FACS at day 20 of differentiation (Figure: 25) and performed a real time analysis in both cell populations. The expression of both, *Tph2* (serotonergic marker) and *Cacna2d1* (candidate gene) was 5 times higher in the GFP-positive (serotonergic) population in comparison to the GFP-negative cells (Figure: 26).

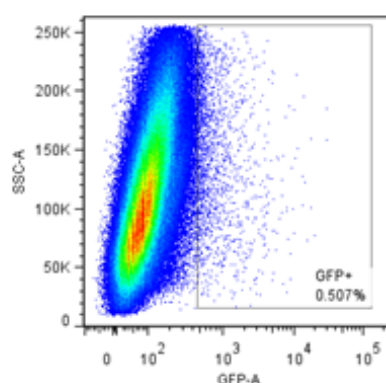


Figure: 25. FACS analysis of *Tph₂::eGFP^(FRT-neo-FRT)* miPS cells after 20 days of differentiation. The GFP-positive population present 0.5% of all cells.

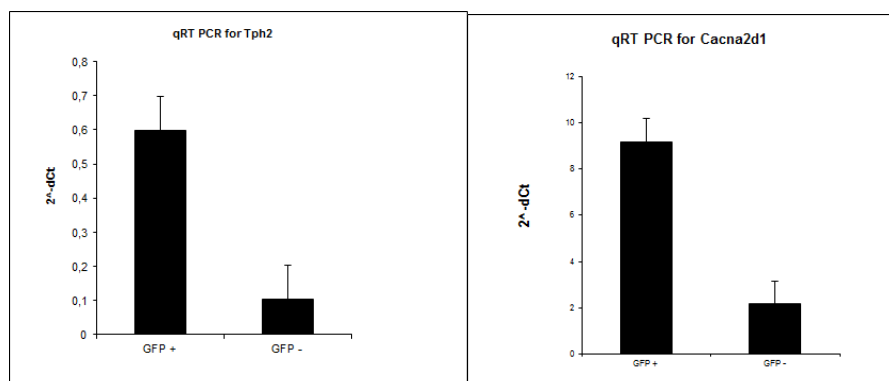


Figure: 26. *qPCR* analysis of *Tph2* and *Cacna2d1* expression in *Tph2::eGFP*^(FRT-neo-FRT) after day 20 of differentiation. Both genes were 5 times upregulated in the GFP-positive population in comparison to GFP-negative cells. Data are averaged from two independent experiments.

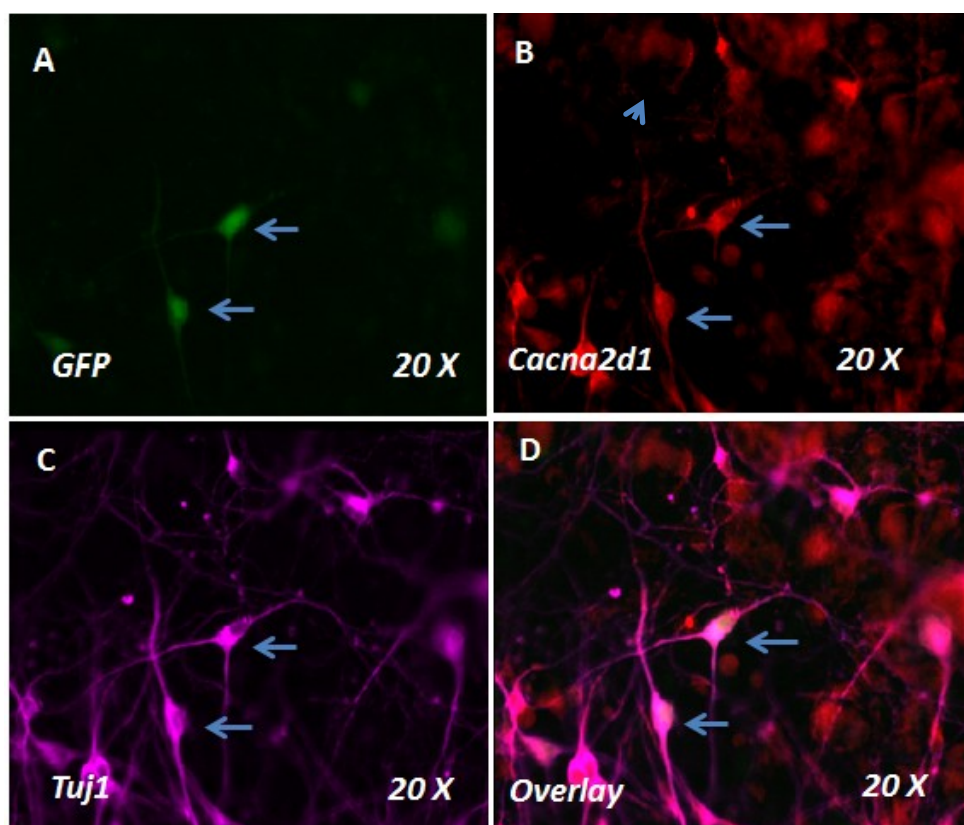


Figure: 27. Differentiation of *Tph2::eGFP*^(FRT-neo-FRT) miPS into serotonergic neurons at day 20. (A) 5-HT positive neurons (green) (B) *Cacna2d1* immunostaining (red) (C) Early neuronal marker Tuj1 immunostaining (violet) (D) Overlay, arrows show cells positive for both *Cacna2d1* and GFP, the arrow head indicates *Cacna2d1*⁺ GFP⁻ neuron.

Next, we checked by immunohistochemistry if *Cacna2d1* is expressed in 5-HT neurons during *in vitro* differentiation of *miPS* cells. This analysis showed that all green (5-HT

positive) cells express *Cacna2d1*, confirming that *Cacna2d1* is expressed in all serotonergic neurons (Figure 27A and B, arrows). However, the *Cacna2d1* expression was not only confined to the 5-HT population but was also detectable in other neurons which were positive for the early neuronal marker *Tuj1* (Figure 27B and C, arrowhead).

4.2.4) Allen brain atlas

Further, to compare the expression of the 5 selected genes with the pattern of expression of *Tph2* in developing mouse brain we used the Allen brain atlas (<http://www.brain-map.org/>). The pattern of expression of *Tph2* and *Cacna2d1* were similar and concentrated in the raphe area of the brain in the Allen brain atlas (Figure: 28). Expression of *Lgi3* was not consistent with *Tph2* (Figure: 20E). This further convinced us to select *Cacna2d1* as our main candidate. *Islr2* and *Ccdc3* were also looked upon but there was no expression seen in the dorsal raphe (Figure: 28), in both coronal and sagittal section of mouse brain. *Itm2b*, because of its ubiquitous expression (Figure: 28J) was not considered further.

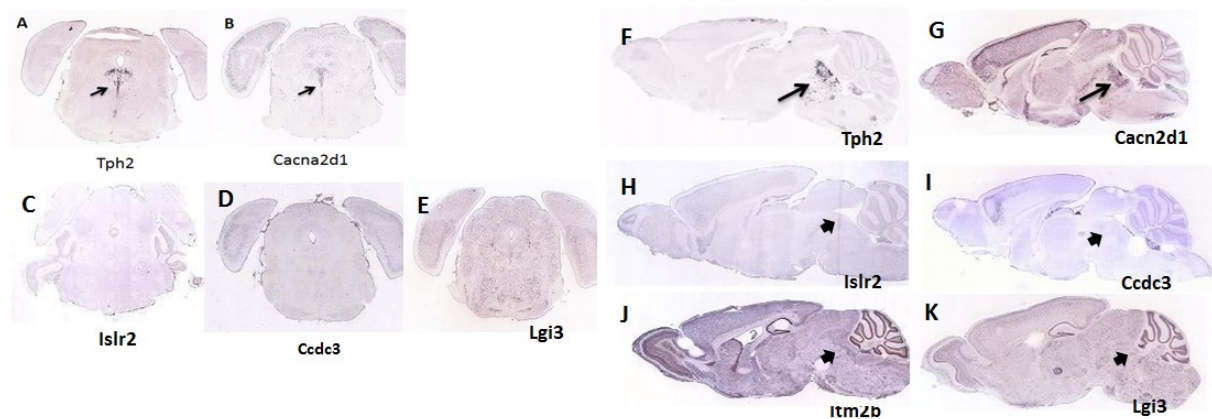


Figure: 28. Expression pattern of *Tph2*, *Cacna2d1*, *Lgi3*, *Islr2*, *Ccdc3* and *Itm2b* (Coronal section for *Itm2b* was not available) in developing mouse brain (P56). ISH expression for *Tph2* is seen to be concentrated in the raphe area of the brain (A). However the expression of *Cacna2d1* (B) apart from raphe (arrow) is also distributed in other areas of the brain. (C) *Islr2*, *Ccdc3* (D) and *Lgi3* (E) was not seen to be expressed at all in the raphe area of mouse brain. In Sagittal sections, *Tph2* and *Cacna2d1* were still concentrated in dorsal raphe (F and G, arrow). There was no expression of *Islr2* or *Ccdc3* or *Lgi3* (H, I and K). *Itm2b* (J) was ubiquitously expressed in the mouse brain.

4.3) *Cacna2d1* and the serotonergic system

Cacna2d1 was selected as prime candidate and was followed upon by characterizing its expression and role in mouse (*Mus musculus*) and zebrafish (*Danio rerio*) animal models. The possibility to alter gene expression and analyse the resulting phenotype in the animal was taken into consideration for the selection of these species. In this chapter, we evaluate if genetic deletion (mouse) or knockdown (zebrafish) of *Cacna2d1* has effects on the development of the animal and on the serotonergic system.

4.3.1) *Cacna2d1* in mouse

To analyse the role of *Cacna2d1* in the development of serotonergic neurons in mouse we selected a previously established knockout model (Fuller-Bicer et al., 2009).

We first evaluated the expression of *Cacna2d1* in mouse brain by western blot and immunohistochemistry.

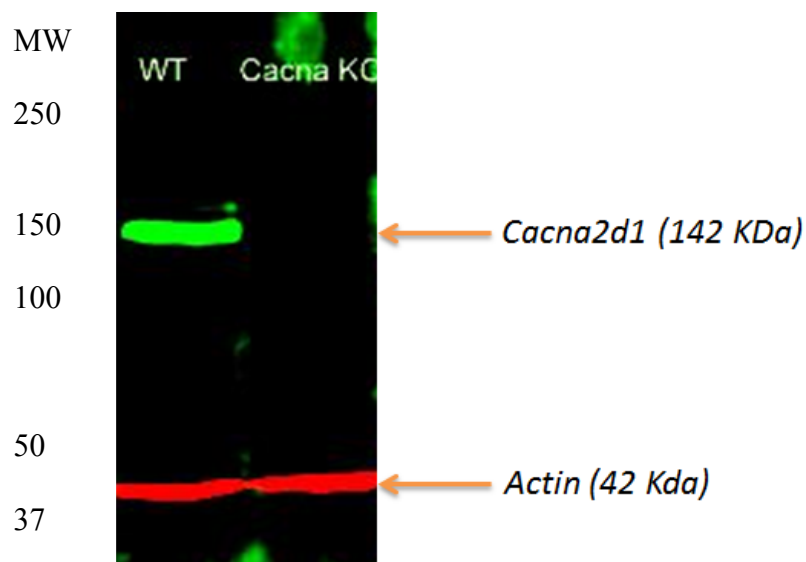


Figure: 29. Western blot analysis of proteins isolated from the hypothalamus of mouse brain. A 142 kDa protein was detected by *Cacna2d1*-specific antibodies. The specificity of the antibodies was confirmed by the absence of the band in the hypothalamus of the *Cacna2d1* knockout mouse.

Western blot analysis using *Cacna2d1* antibodies revealed a single band of the correct length corresponding to the *Cacna2d1* protein. The absence of the band in the hypothalamus of

Cacna2d1-knockout mice confirmed the specificity of the antibodies (Figure: 29). The same antibody was then used to evaluate the distribution of Cacna2d1 protein in various brain areas.

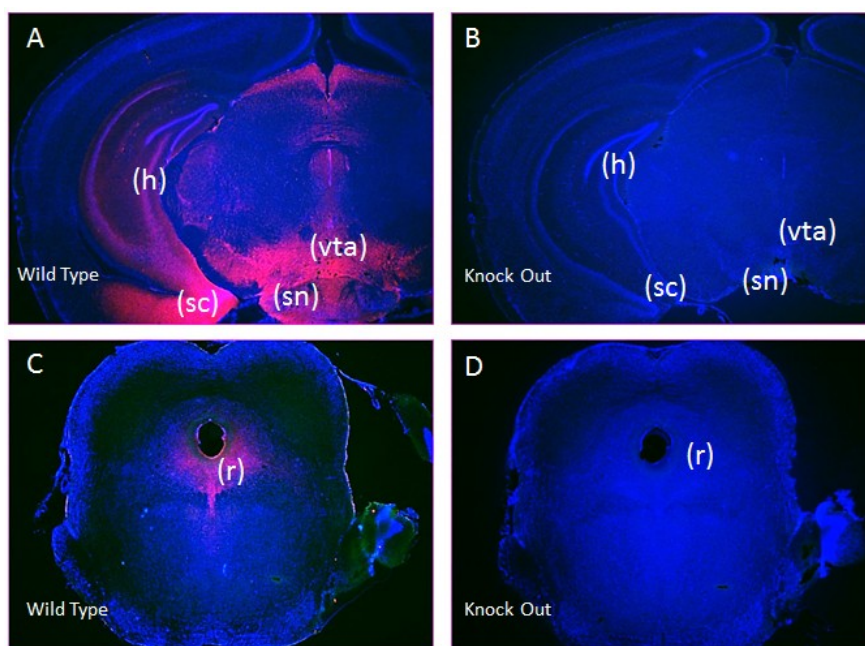


Figure: 30. Representative images of sagittal sections of mouse brain stained with anti-Cacna2d1 antibodies. Strong expression of Cacna2d1 was evident in hippocampus (h), superior colliculus (sc), substantia nigra (sn), raphe nuclei (r) and ventral tegmental area (vta) of wild type mice (A and C) whereas complete loss of Cacna2d1 protein was observed in knockout brain (B and D).

A strong expression of Cacna2d1 was observed in hippocampus, superior colliculus, substantia nigra, and ventral tegmental area (vta) (Figure: 30A). Also, rostral and caudal raphe nuclei were positive for Cacna2d1 (Figure: 30C), confirming the distribution of Cacna2d1 transcripts in the Allen brain atlas. Upon closer look Cacna2d1 was shown to have a similar pattern of expression as that of 5-HT in the raphe area of the mouse brain (Figure: 31A, B).

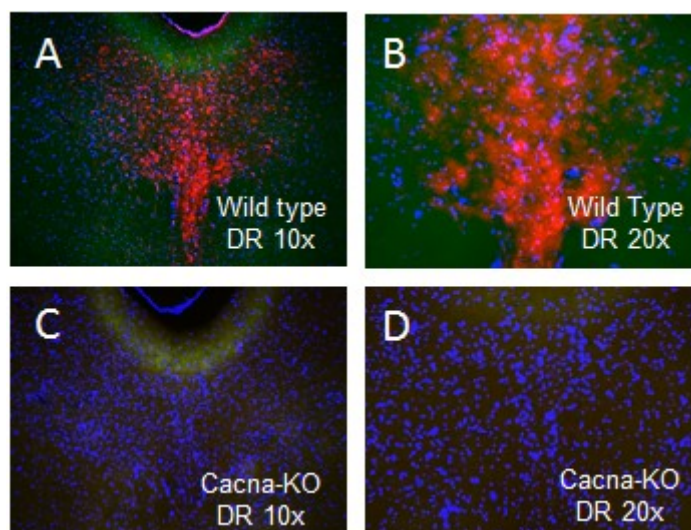


Figure: 31. *Cacna2d1* expression (red) in raphe nuclei of wild type mouse (A and B) vs *Cacna2d1* KO mouse (C and D).

To evaluate if the genetic deletion of *Cacna2d1* affects the development of the serotonergic system we looked for the distribution of 5-HT neurons in *Cacna2d1* knockout mice. Immunohistochemistry with 5-HT antibody was performed in different areas of the brain such as raphe nuclei where serotonergic neurons originate from, and hypothalamus and cortex, areas which receive serotonergic innervations. However, no obvious difference was observed in the distribution of serotonergic neurons between wild type and *Cacna2d1* knockout brains (Figure: 32).

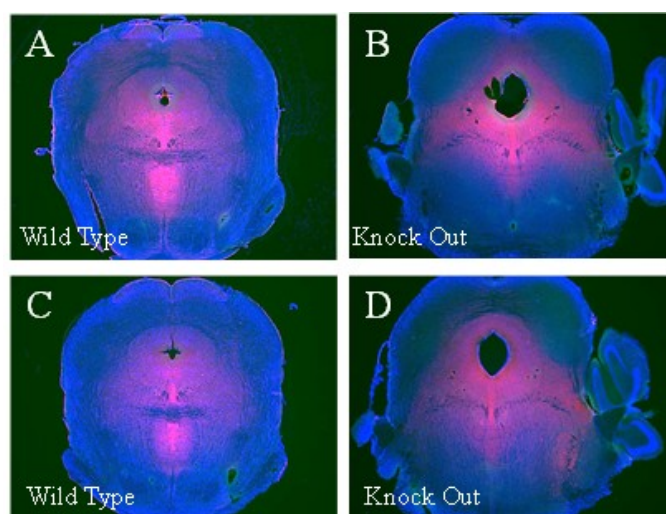


Figure: 32. Representative 5-HT immunostaining (red) in transverse section of brain from wild type (A and C) and *Cacna2d1* knockout (B and D) mice.

4.3.2) 5-HT and *Cacna2d1* in zebrafish

Further in pursuit of looking for the role of *Cacna2d1* in the 5-HT system we went into the zebrafish model. The serotonergic system is highly conserved in vertebrate species, including zebrafish.

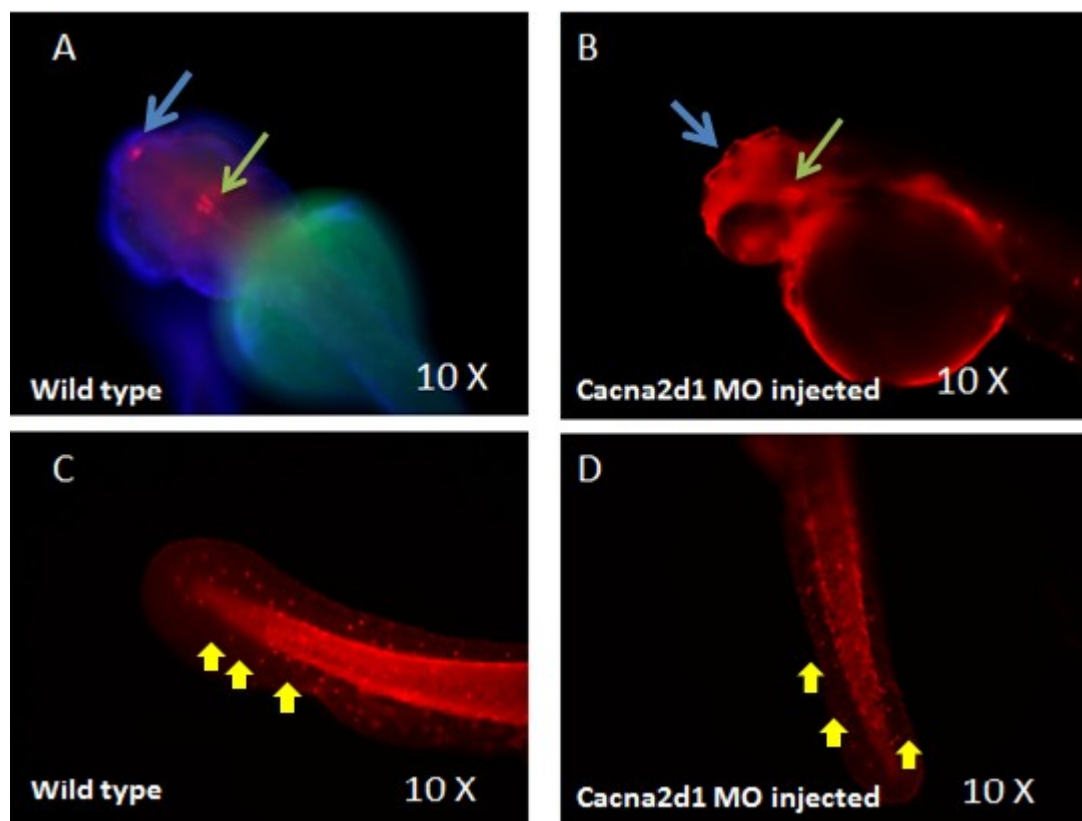


Figure: 33. Representative 5-HT immunostaining (Cy3; Red) in raphe nuclei (green arrow), pineal gland (blue arrow) and in NEC (neuroepithelial cells of skin)(yellow arrow head) in wild type (A, C) and *Cacna2d1* morpholino (MO) treated fish (B, D) on day 3 of development. 5-HT expression was not changed in the raphe upon injection.

In order to look for phenotypic changes in the serotonergic system, we performed immunostaining with anti 5-HT antibodies in wild type zebrafish in comparison to zebrafish injected with *Cacna2d1*-morpholino. Serotonin-positive cells were detected in raphe nuclei of the brain stem, in the pineal gland, and in the neuro-epithelial cells in the skin of wild type zebrafish embryo (Figure: 33C). The distribution of serotonergic cells was unchanged after *Cacna2d1* MO knockdown, suggesting that similar to mouse, serotonergic specification in zebrafish does not depend on *Cacna2d1*.

4.4) *Cacna2d1* in zebrafish development

We next evaluated if *Cacna2d1* plays a role in zebrafish development.

4.4.1) Expression pattern of *Cacna2d1* in the zebrafish embryos

We started with whole mount in situ hybridization to check for the presence of its mRNA level in the embryo. The mRNA expression of *Cacna2d1* was detectable by in-situ hybridization at 48 hpf of development in the notochord which runs centrally from posterior to anterior end. The expression was first observed in cells of the posterior lateral ganglion (pll_g) (Figure: 34A) which is situated right under the developing otic vesicle in the zebrafish.

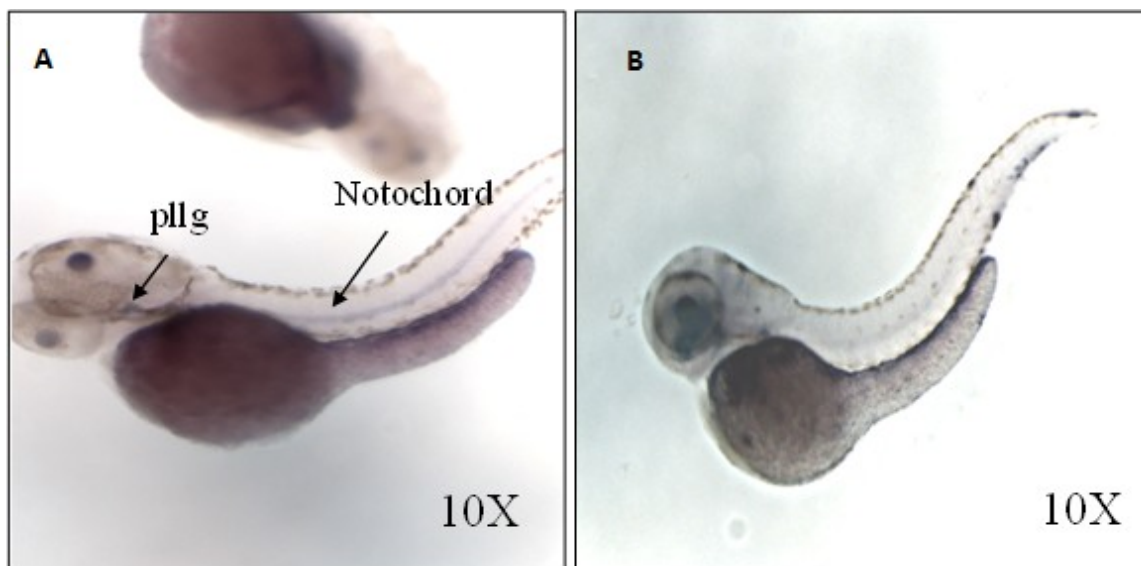


Figure: 34. In situ hybridization of 2 dpf zebrafish embryo with (A) *Cacna2d1* riboprobe and (B) negative control (without *in situ* probe). *Cacna2d1* is expressed in posterior lateral ganglion, notochord and in eyes.

This expression pattern was persistent after day 2 in developing zebrafish.

4.4.2) Whole mount immunostaining of zebrafish embryo

To look for the expression pattern of *Cacna2d1* protein in the developing zebrafish at different developmental stages we performed whole mount immunohistochemistry. The embryos were collected at different time points during the development (8 hpf, D1, D2, D3 and D4) and were stained with *Cacna2d1* antibody. Due to the conserved nature of the protein we used

mouse *Cacna2d1* antibody for the staining. At 8 hpf we could not identify any signal, however the expression of *Cacna2d1* was detected from Day 1 onwards. The *Cacna2d1* protein was present in the areas near the otic vesicle and along the central lateral line which runs along the notochord (Figure: 35).



Figure: 35. Whole mount immunohistochemistry with *Cacna2d1*. Lateral view of (A) Day 2, (B) Day 3 and (C) Day 4 zebrafish embryo. The cells which are positive for *Cacna2d1* are labeled with Cy3 (red), in otic vesicle and along the central lateral line (A), (B) and (C) and in the tail tip (C). Arrow indicates the otic vesicle.

The expression was moving from anterior to posterior part of zebrafish embryo as the embryo developed. Upon looking closely we saw cells which were positive for *Cacna2d1* in the central lateral midline of the zebrafish embryo which were later identified as neuromast cells. (Figure: 36B,F and J).

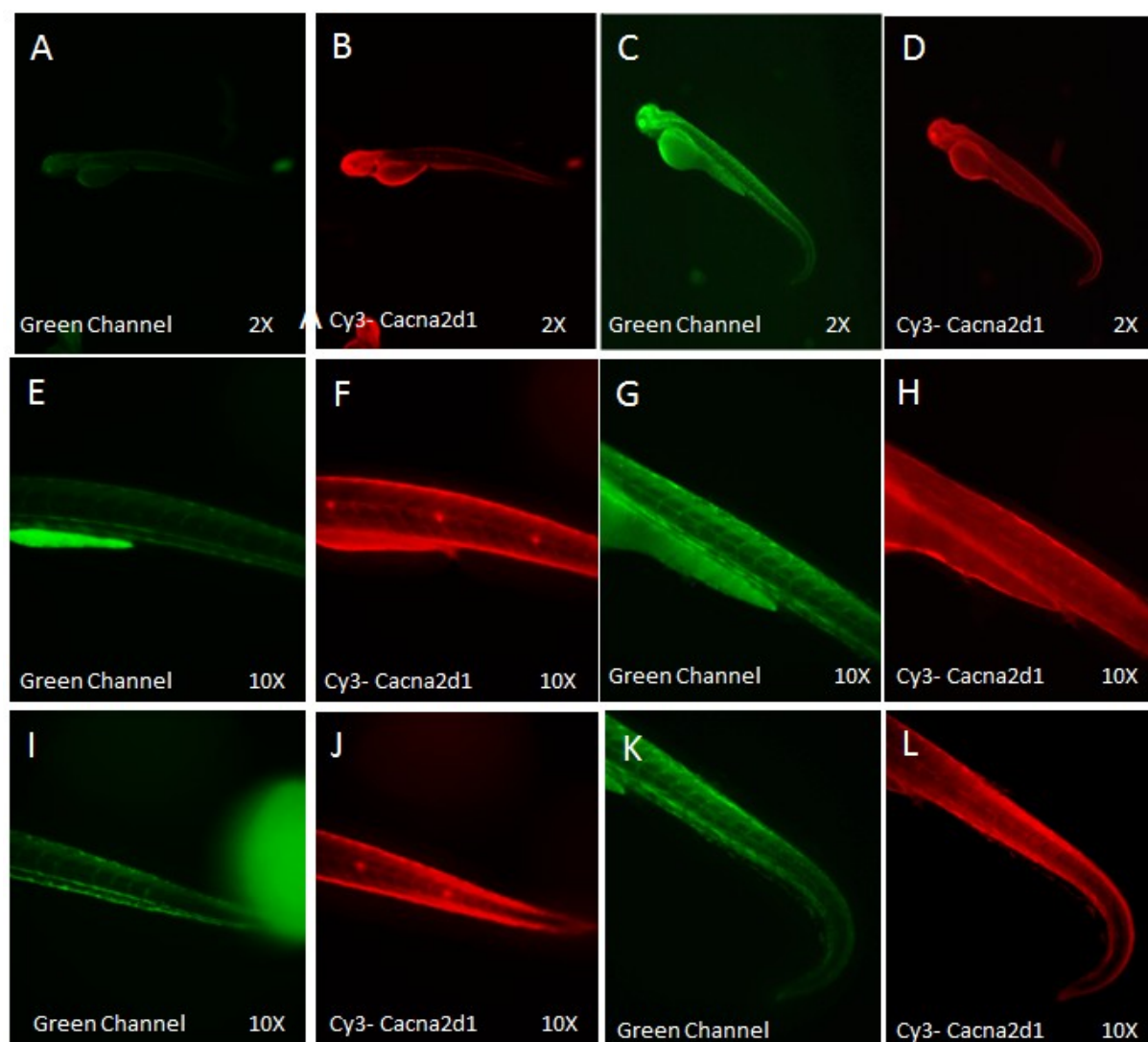


Figure: 36. Whole mount *Cacna2d1* immuno-staining (Cy3, Red) of Day 3 zebrafish WT and MO injected embryo. The expression was concentrated in the central midline of zebrafish embryo (B, F, J) while there was absence of signal in green channel (auto-fluorescence) (A, E, I). The loss of *Cacna2d1* positive red cell upon morpholino injection was clearly seen in trunk region of 3 days old MO injected embryo (D, H) and in the tail (L).

4.4.3) Spatio-temporal expression of *Cacna2d1* in zebrafish embryo

As next step towards characterizing the expression of *Cacna2d1* in zebrafish we performed PCR analysis along the development. After pooling about 50 embryos from different time points (8 hpf, Day 1, Day 2, Day 3, Day 4 and Day 5), embryos were dissected under a binocular microscope into three different parts, namely head, trunk and tail tip (Figure: 37).

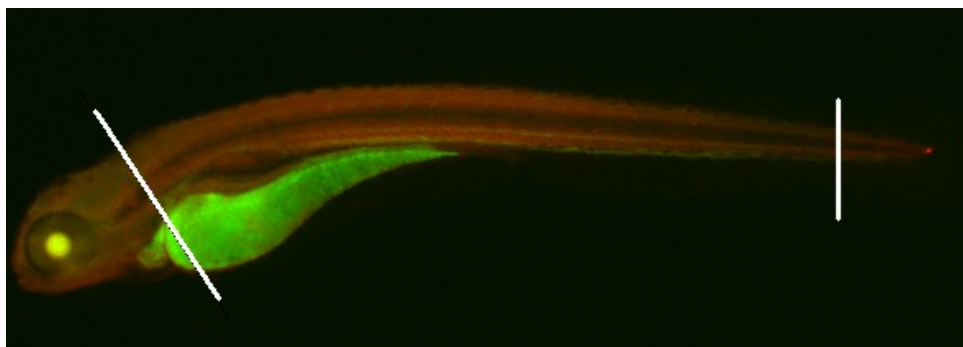


Figure: 37. 3 Days old zebrafish embryo. Zebrafish was dissected into three different parts as shown in the picture, into head, trunk and tail.

RT-PCR analysis showed that in the head (which included pll_g in this case) expression of *Cacna2d1* at transcript level can be seen from day 1 until day 5. In the trunk the expression was detectable from day 3 onwards. In the tail tip expression was detected only at day 5. By that time, there was constant expression of *Cacna2d1* in all the three different parts (Figure: 38).

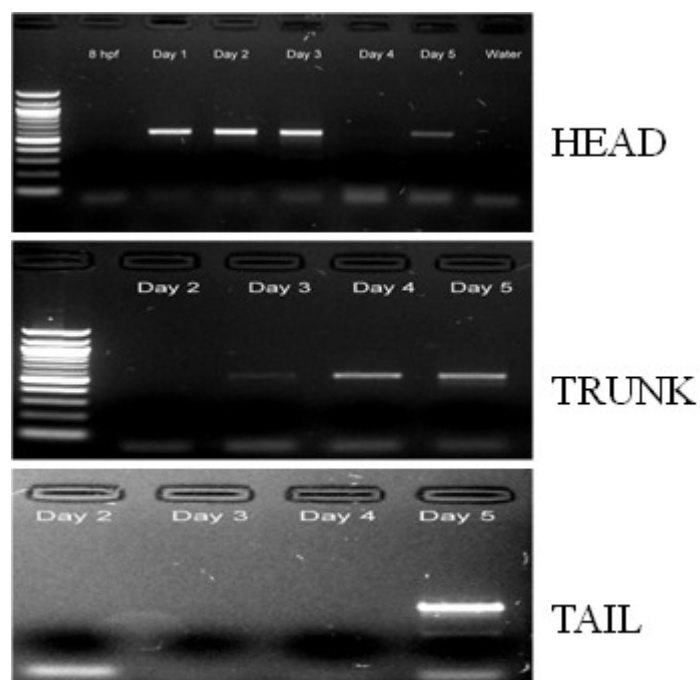


Figure: 38. RT-PCR for *Cacna2d1* mRNA derived from dissected parts of zebrafish embryo at different time points of development ; 8 hpf, Day 1 , Day 2, Day 3, Day 4 and Day 5.

We then looked for the protein expression level of *Cacna2d1* in developing zebrafish by western blot. In accordance with the RNA expression pattern observed by RT-PCR, no

protein expression was detected in 8 hpf whole zebrafish embryo. The expression appeared from Day 1 onwards with increases in protein level along the development (Figure: 39).

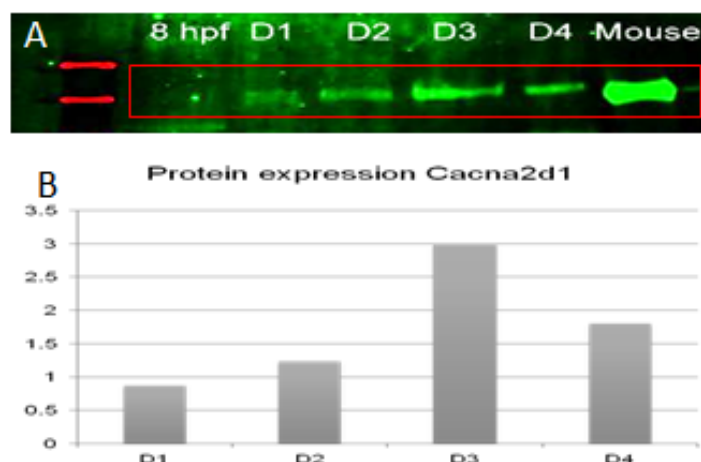


Figure: 39. (A) Western blot analysis of protein extract prepared from whole zebrafish embryo at different days of development, the protein samples were resolved by reducing SDS-PAGE, transferred to PVDF membrane, and probed with an antibody directed against Cacna2d1. Cacna2d1 protein was detected from day 1 till day 4 with increasing in protein concentration. (B) Quantification of Cacna2d1 protein during different days (n=50).

We conclude from this data that there is a spatio-temporal distribution of *Cacna2d1* in the developing zebrafish at both transcript and protein level.

4.4.4) Morpholino (MO) mediated knockdown of Cacna2d1

To further access the functional implication of *Cacna2d1* in zebrafish development morpholino (MO) mediated knock down in zebrafish was performed. MO against *Cacna2d1* was injected into the animal pole of 1-8 cell stage zebrafish embryos. Phenotypic analysis was performed at different time points of development. Based on initial test experiments, the concentration of 150 nmol/μl of MO was selected for the experiments. The concentration of 300 nmol/μl was found to be too toxic and 75 nmol/μl did not show any effect (data not shown).

The MO injected embryos were collected at different time points during their development and analysed using a phase contrast microscope.

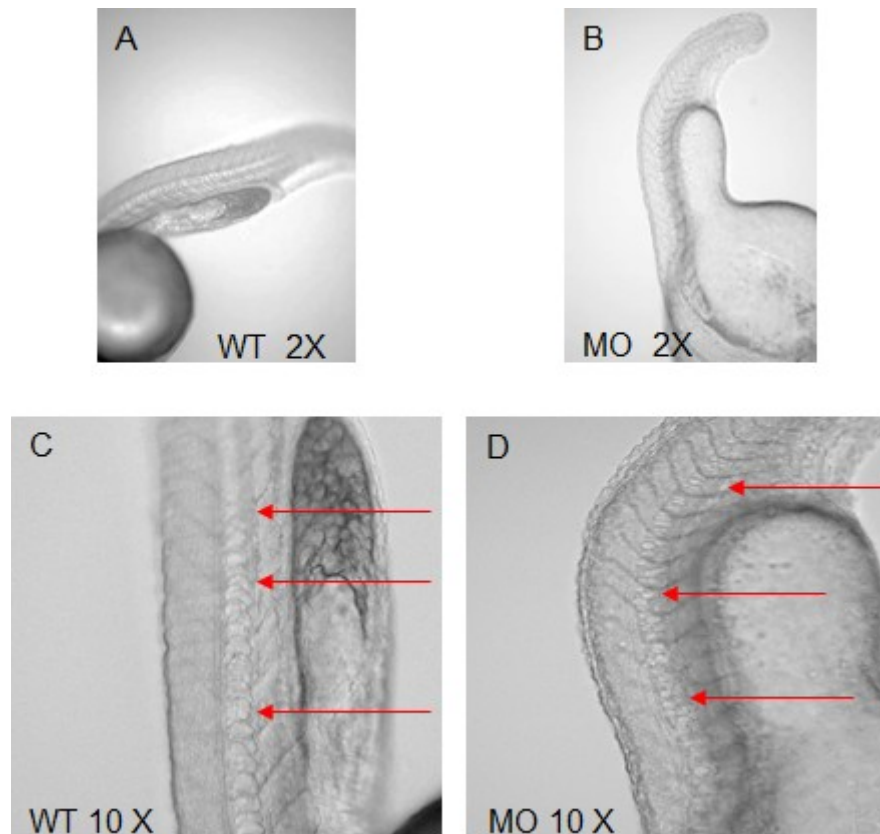


Figure: 40. Phase contrast picture of 1 day old wild type zebrafish embryo (A,C) and embryo injected with anti-sense morpholinos (MO) against *Cacna2d1* (B,D) Red arrows show the disrupted structure of notochord upon morpholino injection in zebrafish as compared to control (A) and (C).

Cacna2d1 MO knockdown induced distinct phenotypic changes in zebrafish. The injected embryos showed peculiar curved shape and were developmentally delayed. Cardiomyopathy was also seen in the injected embryos. The phase contrast imaging of MO injected embryos revealed that the structure of the notochord was disturbed (Figure: 40). In the wild type zebrafish at 26 hpf of development, notochord was seen to be made up of a series of bean shaped cells which later on fuse to form a completely developed notochord (Figure: 40C). However, in the MO knockdown embryos the notochord was made up of a number of circular shaped cells and the normal bean shape of notochord forming cells was gone (Figure: 40D). Also, the knockdown of *Cacna2d1* resulted in the disappearance of a cluster of cells in the central lateral midline of zebrafish (Figure: 36). We hypothesized that these cells are neuromasts. In order to further clarify the nature of these cells as neuromasts and to instigate

the involvement of *Cacna2d1* in the development of these cells we performed DASPEI staining on both wild type and MO injected embryos.

4.4.5) DASPEI staining in wild type and MO mediated knockdown in zebrafish

DASPEI (2-(4-(dimethylamino)styryl) -N-Ethylpyridinium Iodide) is a styryl dye that stains mitochondria of live cells. This dye has a large fluorescence Stokes shift and is taken up relatively slowly. It has been used for the rapid visualization of several distinct classes of epidermal cells *in vivo* and *in vitro*. This voltage sensitive dye specifically labels sensory hair cells of the lateral line (Harris et al., 2003). These sensory hair cells are part of neuromasts which are a major unit of lateral line. The neuromast is a mechanoreceptive organ which allows the sensing of the mechanical changes in water.

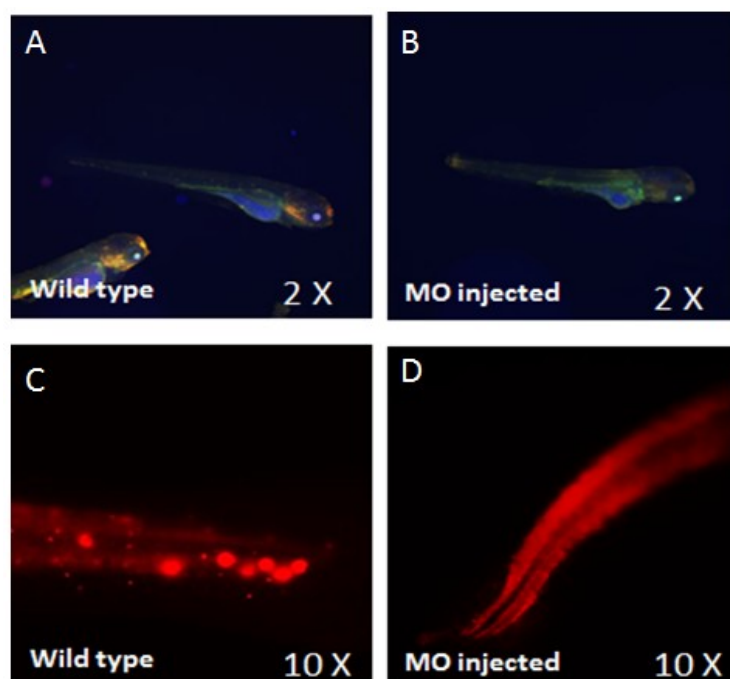


Figure: 41. DASPEI (red) labeling of neuromast. Lateral view of live 4 day old embryo, wild type and *Cacna2d1* MO (A and B). C, D; Higher magnification of the lateral view of tail region. There was clear loss of DASPEI staining in *Cacna2d1* MO injected embryos (D) compared to the terminal neuromast in wild types (C).

In order to check for the effect of *Cacna2d1* MO injection on these neuromasts we performed DASPEI staining. It was observed that after knockdown of *Cacna2d1* there were less

neuromasts in the trunk area followed by a complete loss of neuromast at the tail tip, which are also known as terminal neuromasts. (Figure: 41).

4.4.6) Characterization of *Cacna2d1* in *tg(cldnB:lyn₂GFP)* zebrafish model

To further characterize *Cacna2d1* we analysed the transgenic zebrafish line, *tg(cldnB:lyn₂GFP)*. This line carries a *cldnB:lyn₂GFP* transgene which allows to visualize neuromast cells by GFP fluorescence. We first wanted to know if *Cacna2d1* is expressed in these GFP positive cells. We collected *tg(cldnB:lyn₂GFP)* zebrafish embryo at different time points and performed whole mount immunostaining. We were able to see the co-localization of *Cacna2d1* with GFP positive cells confirming *Cacna2d1* expression in the lateral line of developing zebrafish (Figure: 42).

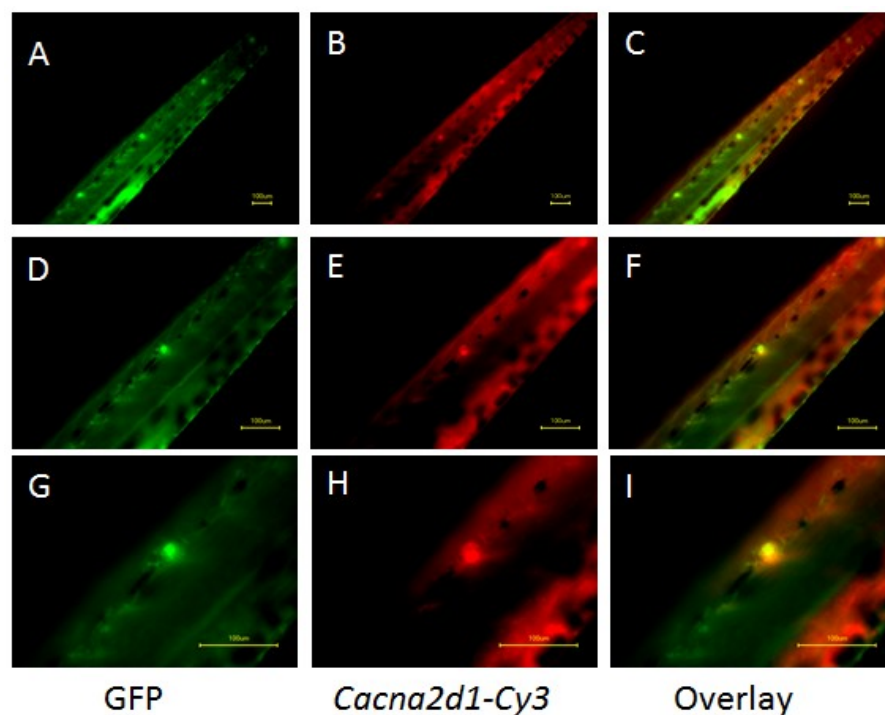


Figure: 42. *Cacna2d1* expression in the central lateral line of 4 day old developing zebrafish. *Cacna2d1* staining (Cy3; Red) (B, E, H) co-incides with GFP positive neuromasts from *tg(cldnB:lyn₂GFP)* line (A, D, G).

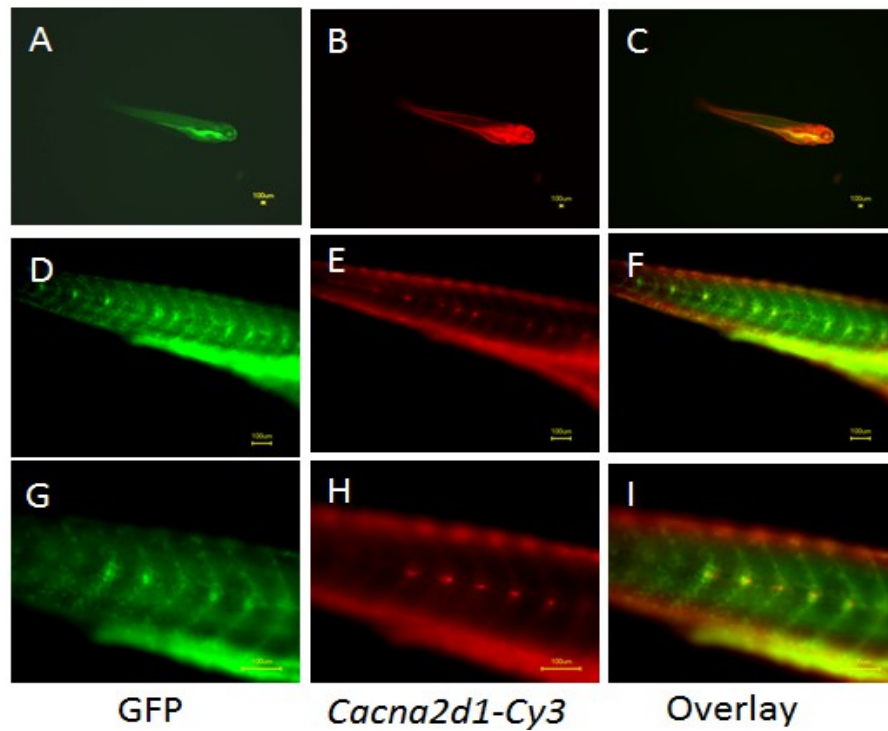


Figure: 43. Disruption in the development and migration of lateral line neuromasts upon injection with *Cacna2d1* MO in *tg(cldnB:lyn₂GFP)* transgenic line. The neuromasts were found to be concentrated in the trunk area (B,E,H. *Cacna2d1* Cy3 staining) with complete lack of neuromast in the tail tip of MO injected 4 day old zebrafish embryos.

4.4.7) Morpholino mediated knockdown of *Cacna2d1* in *tg(cldnB:lyn₂GFP)* zebrafish

To determine the *in vivo* role of *Cacna2d1* in the regulation of neuromast cells in the central midline, *tg(cldnB:lyn₂GFP)* zebrafish embryos were microinjected with *Cacna2d1* specific morpholino. We observed a similar phenotype to that of injected wild type zebrafish. The embryos were curled and showed developmental problems like cardiomyopathy and slow heart rate (Data not shown). A weak expression of *Cacna2d1* (probably because of incomplete knockdown) was still visible in neuromasts (Figure: 43). We observed complete or partial loss of GFP positive neuromast cells upon MO injection confirming the important role of *Cacna2d1* in neuromast development.

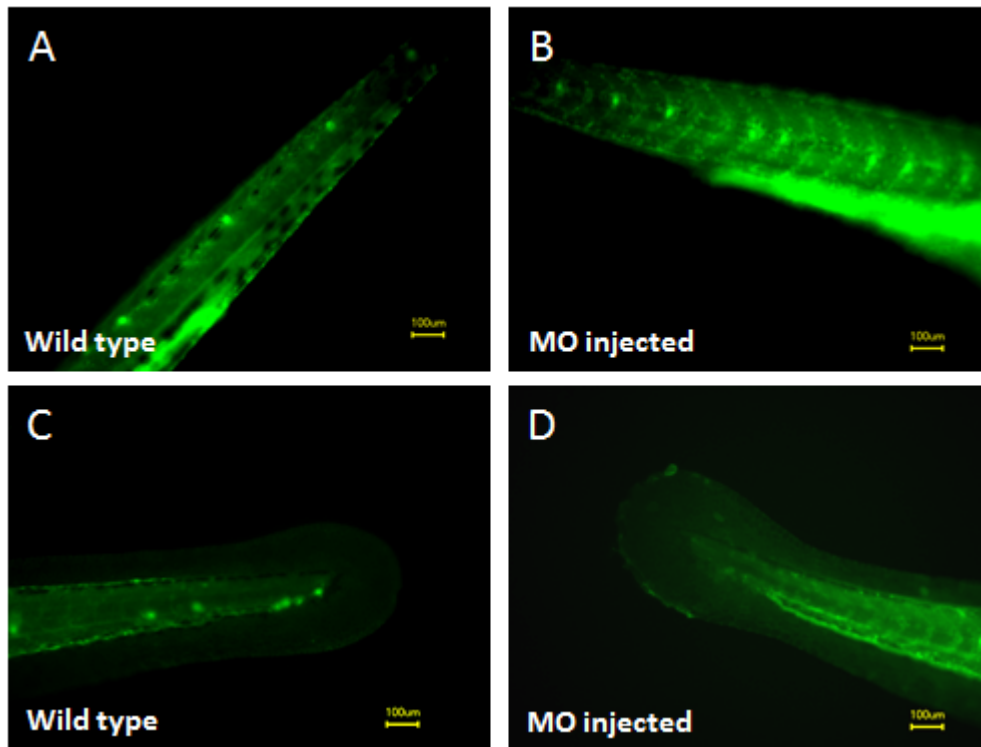


Figure: 44. Pattern of the neuromast deposition in the embryonic posterior lateral line on Day 4 in *tg(cldnB:lyn₂GFP)*. The number and position of the neuromast was analyzed in control zebrafish trunk (A) and tail tip (C) vs. *Cacna2d1* MO injected zebrafish (B and D).

As expected there was a complete loss of terminal neuromasts in the MO injected embryos as compared to un-injected ones (Figure: 44C and D) and other neuromasts were also distributed in an un-organized fashion (Figure: 44A and B). To see the effect of *Cacna2d1* knockdown on *tg(cldnB:lyn₂GFP)* during development we then performed another set of experiments. We collected zebrafish embryos at different time points upon injection of *Cacna2d1* MO and looked for the difference in distribution of neuromast cells in the wild type zebrafish vs. MO injected zebrafish.

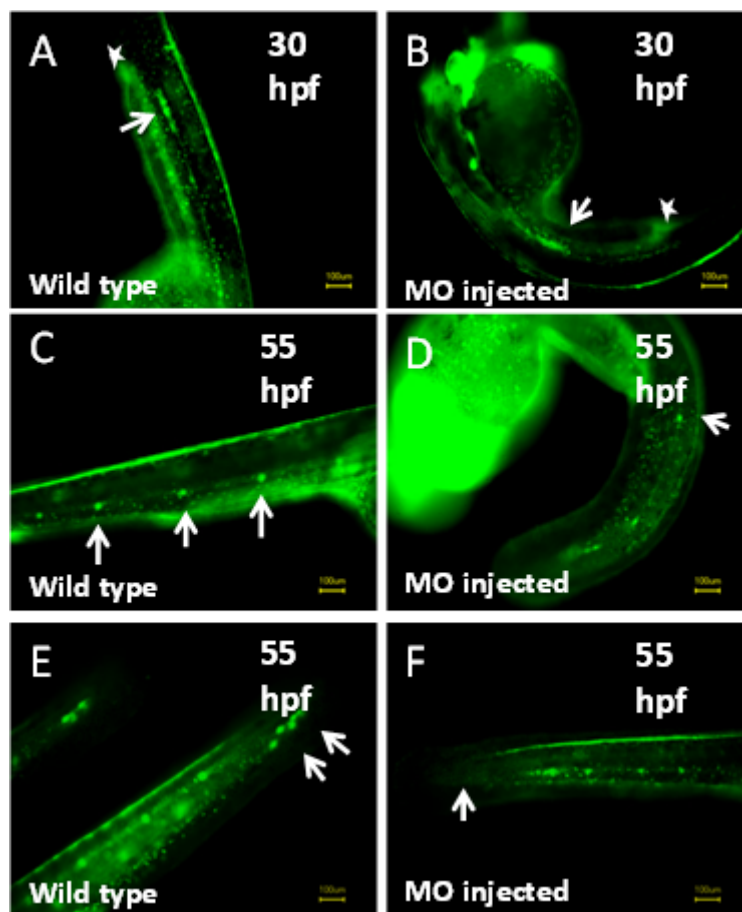


Figure: 45. Migrating primordium in tg(cldnB:lyn₂GFP) wild type vs. *Cacna2d1* MO injected embryo at 30 hpf. The primordium marked by arrow in MO injected embryos (B) was lagging behind the wildtype un-injected embryos (A). At 55 hpf the primordium has already deposited neuromast in wild type embryos (C) including terminal neuromast (E). However the *Cacna2d1* MO injected embryos lacked neuromast deposition and migration to the tail tip (D, F). The anus is marked by a star.

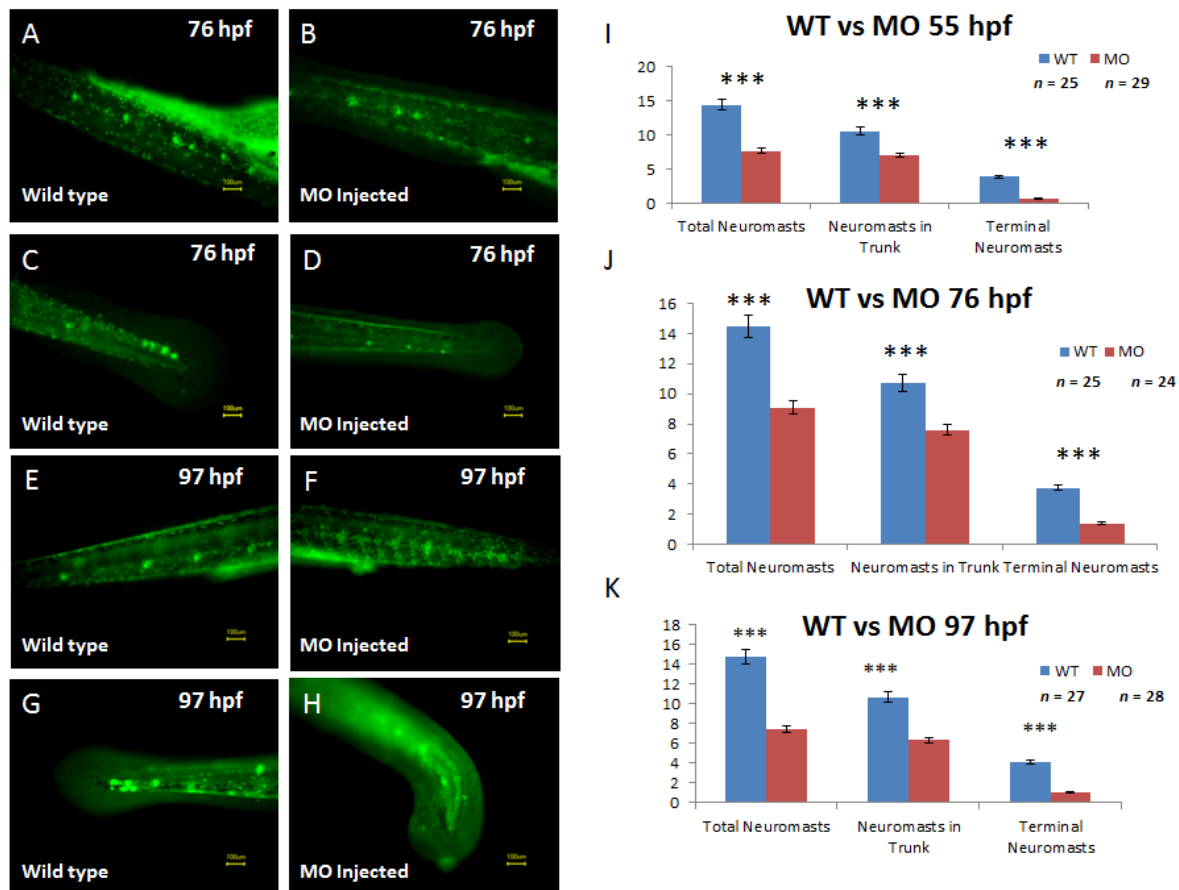


Figure: 46. Lateral line migration in wild type (A,C,E,G) and *Cacna2d1* MO injected (B,D,F,H) *tg(cldnB:lyn₂GFP)* embryos at 76 hpf (A), and at 97 hpf (E). In *Cacna2d1* MO injected fish the neuromast were either absent or not distributed normally (B,D,F). At 97 hpf in wild type *tg(cldnB:lyn₂GFP)* embryo there were four terminal neuromast arranged in a typical manner (G), which were lost upon injection with *Cacna2d1* (H). Number of neuromasts quantified in *tg(cldnB:lyn₂GFP)* embryo at 55 hpf (I), 76 hpf (J) and 97 hpf (K) respectively. There was an overall decrease in the number of neuromasts in the *Cacna2d1* MO injected embryos. *** $P < 0.0001$, WT vs MO.

We collected embryos at 5 different developmental stages in the zebrafish (30, 37, 55, 76, 97 hpf). At 30 hpf the primordium in the wild type embryo had already migrated to the anus (Figure: 45A) whereas, in the injected ones the migration was slower and had only reached the middle of the yolk sac (Figure: 45B). Then at 37 hpf it was clear that the migrated primordium in the wild type *tg(cldnB:lyn₂GFP)* had already moved beyond the end of yolk sac and had deposited at least 3 bunches of neuromasts, whereas in the MO injected fish they were still half way through the yolk sac and had only deposited 2 (not shown). At 55 hpf of development the neuromasts in wild type had already organized themselves (Figure: 45C) and had reached the tip of the tail (terminal neuromasts) (Figure: 45E), in contrast to the MO

injected embryos in which the distribution was random and no terminal neuromast cells could be observed (Figure: 45F). As development progressed the neuromasts in the wild type *tg(cldnB:lyn2GFP)* had already arranged themselves along the lateral line but there was still a lack of arrangement and missing neuromasts in the MO injected embryos (Figure 46D, 46F, and 46H). We then counted the total number of neuromasts, and the number of neuromasts in trunk and tail in the wild type vs. MO injected embryos and found out that there was significant decrease in the number of neuromasts in the embryos that were injected with *Cacna2d1* MO (Figure: 46I, 46J, and 46K), which further showed the involvement of *Cacna2d1* in the development of the lateral line.

5. DISCUSSION

5.1) Rat induced pluripotent stem cells

We generated induced pluripotent stem (iPS) cells in order to differentiate them into serotonin producing neurons and to find the regulators involved in the process of serotonergic differentiation. Over the past few years, *iPS* cells have been a major topic of research. They are seen as one of the options to tackle a number of diseases and are often termed as the future of medicine. In the first part of this work, the approach for the derivation of riPS cells, by the use of various previously reported improvements of iPS/ES-related methods, was optimized. While comparable in terms of derivation efficiency, the approach used in this work offered a better survivability of *riPS* cell clones after subsequent culturing as compared to the previously published non-excisable lentivirus-based serum-free system (Li et al., 2009). Contrary to another report (Liao et al., 2009), it was not possible to generate any viable *riPS* cells using serum containing culture medium, even with serum, which was well permissive, for instance, for mouse *iPS* cell derivation. This signifies the importance of using serum free medium for consistent results with *riPS* cell derivation.

During the time course of this study, another group reported a *riPS* cell derivation protocol that used both REFs and rat neural precursors as starting cell types and relied on serum-containing medium, nonexcisable moloney murine leukaemia virus (MMLV)-based delivery (in combination with ES cell extracts), and mitotically inactivated REFs as feeder cell layer (Chang et al., 2010). However, the approach used in our work seems to offer several advantages: (1) well-known superior reprogramming capacity of HIV-based, compared to MMLV-based retroviral vectors; (2) a true hit-and-run strategy involving lentivirus excision from the rat genome (without any obvious chromosomal rearrangements) and, thus, the elimination of the risks associated with reprogramming factor re-activation and, as a subsequence, altered differentiation properties of riPS cells; (3) this approach also eliminates

the dependence on serum batch variation. Recently proposed virus-free *iPS* cell derivation systems (Li et al., 2011, Lin et al., 2011, Zhouh et al., 2009) remain to be tested in the rat.

Despite rapid progress in the development of novel genomic technologies, such as RNA interference (Kotnik et al., 2009) and zinc-finger nuclease technologies (Jacob et al., 2010), gene targeting via homologous recombination in ES cells currently remains the most straightforward approach in functional genomics. However, this technology has not been available for rat until recently, when rat ES cells have been established (Buehr et al., 2008, Li et al., 2008) and subsequently shown to be amenable to homologous recombination (Meek et al., 2010). In this regard, *riPS* cells may represent a viable alternative to rat ES cells for functional genetics because their derivation, as shown in this study, is rather simple and straightforward and does not require a supply of pre-implantation embryos which can be a quite limited for some strains of rat (Popova et al., 2005), relying instead on easily preserved stocks of rat embryonic fibroblasts. In particular, *riPS* cells can be isolated from the plethora of existing inbred rat models for polygenic diseases (Bader 2010), such as hypertension, diabetes or epilepsy, and used to elucidate the pathogenetic mechanisms involved in these disorders.

After this work, Merkl et al. (Merkl et al., 2013) applied a different approach of generating rat iPS cells using a non-viral inducible vector. By using a single non-viral vector, they were able to have a tighter control over reprogramming. Compared to ES cells, *riPS* cells are preferable for the development of tissue replacement therapies, allowing the isolation of recipient-specific pluripotent cells and overcoming the problem of post-transplantation rejection. Lastly, due to the fact that, to date, models of many human degenerative diseases have been established solely in the rat, *riPS* cells represent a very attractive tool for the validation of therapeutic applications of *iPS* cell technologies in preclinical studies.

5.2) Serotonergic differentiation of pluripotent cells

Next, we aimed at differentiation of ES and iPS cells into serotonin producing neurons. We used different approaches to generate greater populations of serotonin producing neurons from ES cells (refer 4.1.1). We found out that RA treatment lead to the production of a high number of Tuj1-positive neurons, however, the number of 5-HT-positive cells went dramatically down, indicating that RA induction does not favour the differentiation toward serotonergic phenotype, confirming previous studies of our group (Alenina et al., 2006). We also evaluated if application of FGF4 and SHH – two factors which are essential for the formation of 5-HT neurons *in vivo* (Kim et al., 2002) and *in vitro* (Ye et al., 1998) may influence the rate of serotonergic cell formation in our protocol. Although, a slight improvement in serotonergic neuron production was detected in some experiments, no drastic increase in the number of 5-HT-positive cells was observed upon application of these agents. We found out that brief exposure with FGF4 and SHH followed by treatment with bFGF and ascorbic acid gave the best result.

With respect to serotonergic differentiation, numbers of protocols have been developed to enrich the 5-HT population. In one of the recent work, Shimada et al., (Shimada et al., 2010) developed a protocol to induce 5-HT neurons from mouse ES and iPS cells by combining matrigel and noggin. In that work they mimicked coordinated gene expression along the serotonergic lineage *in vivo*, which involves *Mash1*, *Shh*, *Phox2b*, *Nkx2.2*, *Pet1*, *Sert* and *Tph2*. They found that although BMP4 (Bone morphogenic protein 4) abolished the action of noggin, chordin and follistatin could not promote serotonergic differentiation indicating that inhibiting BMP signalling is necessary but not sufficient for serotonergic differentiation. As it has been reported that noggin triggers intracellular calcium increase which, together with neural induction, is inhibited by L-type calcium channel blockers (Bonaguidi et al., 2008). In this respect the appearance of *Cacna2d1*, which is a subunit of the L-type calcium channel, during the differentiation of serotonergic neuron is intriguing (see 5.3.5).

We also tried to differentiate *riPS* cells into serotonergic neurons using our ES cell differentiation protocol but failed to get a 5-HT population. With respect to differentiation, in the recent work Merkl et al. (Merkl et al., 2013), managed successfully to generate *riPS* cells and differentiate them into endodermal, ectodermal and mesodermal lineages, however directed differentiation of *riPS* cells was still not feasible, suggesting that *riPS* cells may require the addition of other inductive transcription factors. However, we managed to differentiate the mouse iPS cells into 5-HT producing neurons using the same protocol that we used for ES cell differentiation (Figure: 24). This further shows that although the generation of both rat and mouse iPS cells is possible by same transcriptional master regulators, the process of differentiation in these two models work via different signalling cascades.

5.3) Factors involved in serotonergic differentiation

In this part of the work, we concentrated on the underlying genetic factors and on the regulators which might be involved in the genetic predisposition to serotonergic differentiation. In order to do that, we started by looking into the process of differentiation of serotonergic neurons from ES cells. The idea was to look for new early genes in the genetic cascade and identifying them along with already existing known genes. We first looked at the transcript level during the differentiation of ES cell into neurons. Three independently carried out experiments provided us with a multitude of data. The first data set was acquired from one of our collaborators from FunGenES (see results). Cluster analysis of the microarray data of ES cell differentiation into neuronal population gave us a cluster which was enriched by all the major serotonergic marker genes like *Tph2*, *VMAT*, *FEV* and *SERT* (Figure: 14). We called it “*Tph2* cluster”.

Lmx1b is a key upstream transcription factor that is involved in the determination and specification of serotonergic neurons (Ding et al., 2003, Pattyn et al., 2004). Deletion of *Lmx1b* in mice leads to the absence of 5-HT neurons in the brain (Zhao et al., 2006, Cheng et

al., 2003). It is also expressed in developing 5-HT neurons with *Pet1* starting around day E11 in the rostral cluster of serotonergic differentiation and later in the caudal region (Cheng et al., 2003). The forced expression of *Lmx1b* was instrumental to generate serotonergic rich population. RNA was collected at different time points during differentiation of this *Lmx1b* over expressing cell line and affymetrix analysis was performed. This served as the second data set.

In 2010, Deneris et al. published a paper titled “Distinct transcriptomes define rostral and caudal serotonin neurons”, which was targeted towards enhancing the understanding of the molecular architecture of serotonergic neurons. In this study, post mitotic serotonin containing neurons were isolated by FACS and affymetrix analysis was done. They identified several hundred transcripts encoding homeodomain, axon guidance, cell adhesion, intracellular signalling, ion transport, and other proteins associated with various neurodevelopmental disorders and showed that they were differentially enriched in developing rostral and caudal 5-HT neurons. The differential enrichment of gene sets for different canonical pathways and gene ontology categories provided additional evidence for the heterogeneity between rostral and caudal 5-HT neurons. The microarray data was taken as the third data set (Deneris et al., 2010). Upon carefully scrutinizing the enriched microarray data sets from these three independent experiments we found 5 novel genes along with *Tph2* and *Pet1*, that were overlapping in all the three studies (Figure: 15). The candidate genes selected were as follows:

5.3.1) Lgi3 (Leucine-Rich Repeat LGI family, member 3)

Leucine-rich glioma inactivated 3 is secreted protein and a member of LGI/epitempin family which consists of four members. The LGI family members share high amino acid sequence homology of 60-70% and have leucine rich repeats (LRR) and epitempin (EPTP) repeats in their C-terminal half regions (Scheel et al., 2002). Lgi3 is shown to be highly expressed in the brain and to play a regulatory role in neuronal exocytosis and differentiation (Gu et al., 2002). In the developing mouse brain, *LGI3* mRNA is expressed at low level and its expression is

markedly increased in broad areas after birth (Lee et al., 2006). Also, intracellular LGI3 has been found to be associated with syntaxin 1 at neuronal synapses which may regulate exocytosis and the secreted LGI3 may initiate neurite growth and neuronal differentiation (Park et al., 2010).

5.3.2) *Islr2* (Immunoglobulin super family containing leucine-rich repeat, also known as *Linx*)

This gene is expressed in a subset of developing sensory and motor neurons. Domain and genomic structures of Linx and other LIG (Leucine-rich repeat and immunoglobulin) family members suggest that they are evolutionarily related to Trk receptor tyrosine kinases (RTKs). Several LIGs, including Linx, are expressed in subsets of somatosensory and motor neurons. LIG family RTKs-associated proteins modulate growth factor signals during neural development (Mandai et al., 2009). *Islr2* is shown to be expressed in a subset of DRG sensory and spinal motor neurons and physically interact with RTKs, including TrkA and Ret. Also, *Islr2* mutant mice exhibit sensory and motor neuron axonal projection defects (Mandai et al., 2009).

5.3.3) *Itm2b* (integral membrane protein 2B, also known as *BRI2*)

The gene is connected to Familial Danish Dementia (FDD) and Familial British Dementia (FBD) causing amyloid and pre-fibrillar effects similar to those seen in Alzheimer Disease (AD). It functions as a protease inhibitor and plays a role in APP processing regulating the physiological production of the beta amyloid peptide. Furthermore, recently *Itm2b* was found to co-localize in retina with the APP (Audo et al., 2014).

5.3.4) *Ccdc3* (Coiled-Coil domain containing protein 3)

The *CCDC3* gene was assigned to chromosome 13. It consists of three exons comprising 2599bp encoding for a protein of 274 amino acids. The strong *CCDC3* sequence homology on amino acid level between species suggests a conserved universal function of this protein (Eberlein et al., 2010). *CCDC3* is a secreted protein. It has been shown to have increased

expression in visceral adipose tissue in abdominally obese subjects. It has also been suggested as a potential biomarker for estimating visceral adiposity (Ugi et al., 2013).

5.3.5) *Cacna2d1* (Calcium channel, voltage-dependent, alpha 2/delta subunit 1)

This gene encodes a member of the alpha-2/delta subunit family, a protein in the voltage-dependent calcium channel (VDCCs) complex. L-type voltage dependent Ca^{2+} channels mediate the influx of calcium ions into the cell upon membrane polarization and consist of a complex of α_1 , α_2/δ , β , and γ subunits in a 1:1:1:1 ratio. It was determined from protein sequencing that α_2 and δ are the product of a single gene (De Jongh et al., 1990).

The pore forming subunit of Ca_v channels is the α_1 subunit, which determines the main biophysical and pharmacological properties of the channel. For the low-voltage-activated (LVA) calcium channels (Ca_v3 or T-type channels), they function well as monomers. However, for the high-voltage activated (HVA) Ca_v1 and Ca_v2 subfamilies, the α_1 subunit is generally thought to be associated with a membrane-anchored, predominantly extracellular, $\alpha_2\delta$ subunit and a cytoplasmic $\text{Ca}_v\beta$ subunit (Bauer et al., 2010) (Figure: 47). The α_1 subunit was identified to be the protein that binds to 1,4-dihydropyridines, which are Ca_v channel antagonists.

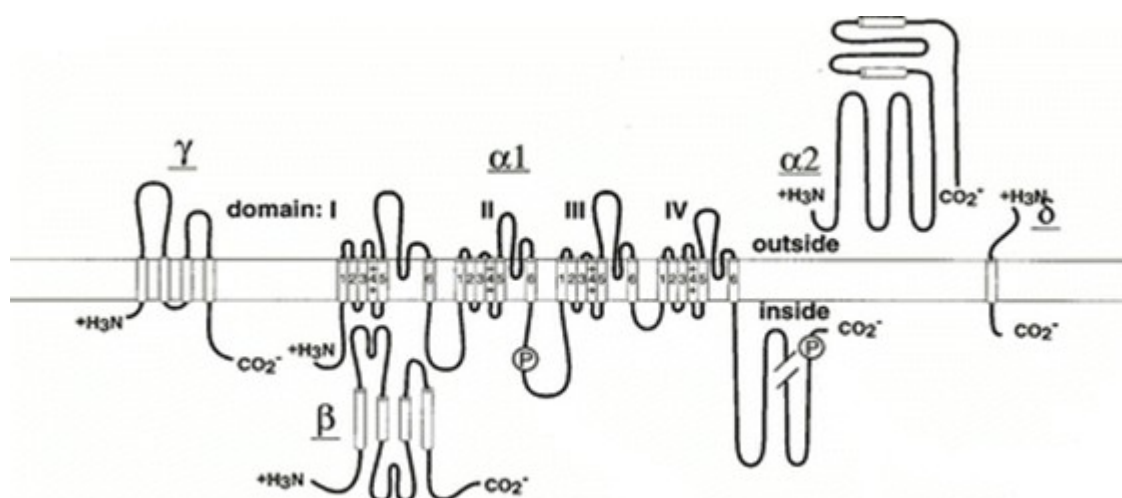


Figure: 47. The subunit composition and structure of Ca^{2+} channels (Catterall et al., 2003)

Mutations in genes coding for $\alpha 2/\delta$ - subunits of VDCCs are known to be associated with diseases (Yaksh 2006). A spontaneous mutation in the *Cacna2d2* murine gene resulted in the Ducky mouse demonstrating signs of epilepsy (Brill et al., 2004). A mutation in the *Cacna2d4* gene introduced a premature stop codon that truncated one third of the protein and led to a cone-rod dysfunction in the visual system of mice (Wycisk et al., 2006). Also, a mutation in the *Cacna2d* gene (*unc-36*) in *Caenorhabditis elegans* has been identified, manifesting in a lack of adaptation (desensitization) to dopamine and serotonin (Schafer et al., 1996).

The gene encoding the $\alpha 2/\delta 1$ subunit (*Cacna2d1*) was originally cloned from skeletal muscle (Ellis et al., 1988) and its expression is found to be ubiquitously distributed (Klugbauer et al., 2003). It is expressed at high levels in brain, heart, skeletal and smooth muscle (Gong et al., 2001). The expression the $\alpha 2/\delta 1$ subunit has been shown to be up regulated in response to spinal cord injury and neuropathic pain (Xiao et al., 2002, Yaksh 2006).

To investigate the consequences of $\alpha 2/\delta 1$ ablation, a conventional knockout mouse using a construct targeting exon 2 of *Cacna2d1* was generated (Fuller-Bicer et al., 2009). The deletion of *Cacna2d1* in mice leads to the loss of the $\alpha 2/\delta 1$ protein in mouse heart, brain, and skeletal muscle and also reduced gabapentin binding in heart. Gabapentin also known as Neurontin, is a pharmaceutical drug that interacts with VDCCs in cortical neurons. Also, since it is known that $\alpha 2/\delta 1$ is associated with different Ca^{2+} channel pore forming $\alpha 1$ -subunits, such as $\text{Ca}_v2.1$, $\text{Ca}_v2.2$ and $\text{Ca}_v2.3$ to form P/Q-, N-, and R-type Ca^{2+} channels (Catterall 2000, Klugbauer et al., 2003), the expression of these subunits was also checked and was found to be normal and not influenced by the knockout. However, the *Cacna2d1* knockout mouse model has not been investigated for brain abnormalities. In this study we used this mouse to study the involvement of *Cacna2d1* in the 5-HT system.

5.3.5.1) *Cacna2d1* and 5-HT system

In this part of study we concentrated on exploring the role of *Cacna2d1* gene in development and differentiation of 5-HT neurons. VDCCs, are present in excitable cells, like neurons, and function by responding to polarization and depolarization of the membrane potential by allowing Ca^{2+} entry or exit, respectively. This in turn provides Ca^{2+} for many processes, including neurotransmitter and hormone releases and also plays a role in calcium dependent gene transcription (Dolphin., 2012). Evaluation of *Cacna2d1* expression during differentiation of stem cells revealed that with increase in the neuronal population there was also a consistent increase in the transcript level of *Cacna2d1* (Figure: 16). Also upon performing immunohistochemistry it was evident that the expression of *Cacna2d1* was localized in the neurons positive for 5-HT (Figure: 27). However, we found that the expression was not exclusive to 5-HT producing cell. This was evident from the fact that *Cacna2d1* was also clearly present in 5-HT negative cells, expressing the early neuronal marker *Tuj1* (Figure: 27). Previously, in 2003 it was shown that the $\alpha 2/\delta 1$ subunit is mainly localized to synapses, rather than cell bodies (Peterson et al., 2003) and it was present on the plasma membrane which was confirmed by electron microscopy. Also, it was found that in DRG neurons, $\alpha 2/\delta 1$ subunits are trafficked from the site of synthesis in the cell body down to the peripheral as well as central axon (Bauer et al., 2009). This suggests that $\alpha 2/\delta 1$ might also have effects on other processes than calcium channel trafficking such as axonal regeneration and sprouting at sites of peripheral injury of DRG neurons (Dolphin., 2012). In accordance with these data, we saw a rise in transcript level only after day 10 of *in-vitro* differentiation which also coincides with the growth of axons and projections from cell bodies. Since it is known that $\alpha 2\delta 1$ overexpression increases synaptogenesis *in-vitro* and *in-vivo* (Eroglu et al., 2009), it further strengthens the idea that the function of *Cacna2d1* is mainly related with synapses, rather than cell bodies (Peterson et al., 2003). This does bridge the possibilities of the two systems

(calcium signalling and 5-HT system) intertwining and working synchronously and maybe even influencing each other.

In this work, we confirmed that *Cacna2d1* is strongly expressed in neurons, both in the 5-HT neuronal population and in the non 5-HT population. The exclusiveness of *Cacna2d1* with serotonergic neuron is still not clear and needs to be further investigated. However, we were able to prove higher expression of *Cacna2d1* in FACS sorted serotonergic cells as compared to the non-serotonergic ones (Figure: 27). We then went ahead into two different animal models, mouse and zebrafish, to look for the direct influence of *Cacna2d1* on the 5-HT system. The *Cacna2d1* knockout mouse model is well established and it has been shown that ablation of α_2/δ_1 subunit did not have any effect on the expression level of other Ca^{2+} channels (Fuller-Bicer et al., 2009). This makes the model an ideal system for studying the α_2/δ_1 subunit since there is no compensatory mechanisms that could possibly compromise the relevance of the data (Fuller-Bicer et al., 2009). However, we did not see any difference in the expression pattern of 5-HT in *Cacna2d1* knockout mice. Serotonergic neurons had a normal distribution in the raphe and in the areas of 5-HT projections like hypothalamus, cerebral cortex and cerebellum (Figure: 33).

Secondly, we looked into the zebrafish model. There were several reasons for selection of zebrafish as a model organism for this study. Among few, its short life cycle and short generation time, fast development, the fact that embryos are fertilized externally and develop outside the female (*ex utero*), its translucent embryos which make it possible to easily observe their development and no previous information about the role of *Cacna2d1* in development, stood out. After knocking down *Cacna2d1*, using morpholino injections in zebrafish embryos we did not see any phenotypical changes in the expression pattern of 5-HT (Figure: 33). This further suggests that *Cacna2d1* may not have a direct influence on the differentiation of 5-HT neurons however the physiological and developmental role of *Cacna2d1* in these cells needs to be resolved.

5.3.5.2) *Cacna2d1* in zebrafish development

The role played by VDCCs in neural transmission has been the subject of intense investigation since first studied in the frog neuromuscular junction (Katz and Miledi, 1967). Of particular significance has been the distinction between the calcium channel isoforms mediating synaptic transmission at certain peripheral and central synapses. A number of studies on L-type Ca^{2+} (LTCCs) channels have shown that they influence the bulk of voltage gated Ca^{2+} entry in vertebrate inner ear hair cells and are essential for mammalian auditory processing (Beutner et al., 2001, Debener et al., 2002). It has been previously reported that LTCC currents are implicated in neurotransmitters release at the hair cell afferent active zone, the ribbon synapse. It is also well established that release of neurotransmitter at the inner hair cell afferent synapses is a fundamental step in translating sound into auditory nerve excitation (Sewell et al., 1996, Beutner et al., 2001).

In zebrafish detection of sound and head acceleration depends on the transduction of mechanical force into electrical signal in hair cells. These hair cells detect and process sound and movement information, and transmit this with remarkable precision and efficiency to afferent neurons via specialised ribbon synapses (Olt et al., 2014). In zebrafish lateral line, these mechanosensory hair cells together form individual sensory organs, the neuromast. In this study we found that, the *Cacna2d1* is present in the neuromasts (Figure: 36, Figure: 42). We also showed that loss of *Cacna2d1* gene leads to developmental problems and disturbance in lateral line migration of neuromast cells (Figure: 43, Figure: 45).

The first step in the posterior lateral line development is the migration of the primordium along the horizontal myoseptum that separates dorsal from ventral somatic muscle. *In-situ* hybridization revealed that *Cacna2d1* specific notochord expression in lateral midline of zebrafish starts from day 2 onwards, indicating an involvement of *Cacna2d1* in development of the lateral line (Figure: 34). Also, we saw that *Cacna2d1* is expressed in a spatio-temporal manner. The expression of *Cacna2d1* was not only concentrated in the central midline, but

specifically in the migrating neuromasts. The expression was also up-regulated both at transcript and protein level, indicating the role of *Cacna2d1* in development (Figure: 38, Figure: 39). In order to quantify the number of neuromast clusters in *Cacna2d1*- MO injected and wild type fishes, we examined embryos at different time points after staining with 4-Di-2Asp. Lateral line advancement towards tail gets over by 30 hpf, however it takes between 48-96 hpf for terminal neuromasts to form. We counted neuromasts at 76 hpf and 96 hpf (Figure: 46). A direct comparison between control and injected fishes at 96 hpf showed outright loss of terminal neuromasts in injected ones with a number of structures absent along the trunk. Then we scored the number of neuromasts at specific time points at anterior and posterior positions along the body (Figure 46I, 46J and 46K) and saw a decrease in their number. This demonstrated that morpholino induced knockdown of *Cacna2d1* in zebrafish embryos disrupts cell migration and neuromast deposition in developing zebrafish, which further extended into movement defect upon ablation. We also found that the knockdown of *Cacna2d1* in zebrafish embryos leads to reduced spacing of deposited neuromasts and results in premature termination of the pLL system.

With respect to development of zebrafish lateral line, previously, it has been demonstrated that Wnt/ β -catenin signalling stimulates the proliferation of supporting cells in neuromasts of pLL, both during initial development and later in mature neuromast (Head et al., 2013). *Wnt* is a zygotic effect gene, which is expressed during very early embryonic development. It regulates cell fate in the blastula stage, orchestrates morphogenetic movement during gastrulation, and potentiates organogenesis (Antara De, 2011). Members of the vertebrate Wnt family have been subdivided into two functional classes according to their biological activities. Some Wnts signal through the canonical Wnt-1/wingless pathway by stabilizing cytoplasmic β -catenin. On the other hand, activation of the Wnt/ Ca^{2+} pathway results in intracellular Ca^{2+} release, and activation of the Ca^{2+} -sensitive enzymes Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) and protein kinase C (PKC) in a β -catenin-independent

manner (Sheldahl et al., 1999, Khul et al., 2000). Earlier research in *Xenopus* showed that *wnt5a* signalling affects early development of embryo. Later it was also discovered that Ca^{2+} is the secondary messenger in *wnt5a* signalling, which arises from G-protein-linked phosphatidylinositol signalling (Slusarski et al., 1997). It was concluded that the Wnt/ Ca^{2+} signalling pathway is essential for ventral cell fate during early embryogenesis (Khul et al., 2000).

It has been previously shown that loss of the *Cacna2d1* subunit leads to loss of functionality of calcium channels (Templin et al., 2011) and this may impair Wnt signalling. Indeed it was also shown that the modulation of Ca^{2+} release is critical during early embryonic pattern formation and acts, in part, by influencing Wnt/ β -catenin signaling. (Westfall et al., 2003, Park et al., 2005, Asuthkar et al., 2012). Thus the loss of *Cacna2d1* may cause the impairment of pLL migration by deficient Wnt signalling (Head et al, 2013).

Given the role of calcium as an intracellular messenger, it is not very surprising that alteration of the spatial and temporal balances of intracellular Ca^{2+} can influence the developmental process. In accordance with the role of calcium channels in zebrafish lateral line development that we saw in this work, it was reported recently that Calnexin and Calreticulin, two different proteins, which are involved in the regulation of intracellular Ca^{2+} homeostasis, were found to be expressed in the lateral line system (Hung et al., 2013). This further proves the importance of calcium channels and its subunits in the development of the zebrafish lateral line system. Development of nascent neuromasts, or ‘proto-neuromasts’, is initiated by FGF signalling in the migrating PLLp (Lecaudey et al., 2008; Nechiporuk and Raible, 2008). It is also known that *Fgf3* and *Fgf10* are expressed in response to Wnt signalling in a leading domain of the PLLp (Aman and Piotrowski, 2008). Yet, the FGFs are not very effective in activating FGF receptor (FGFR) signalling in the leading domain, at least in part because factors that determine FGF expression also determine the expression of *Sef* (Il17rd – Zebrafish Information Network), an inhibitor of FGFR signalling (Aman and Piotrowski, 2008; Tsang et

al., 2002). As a result, FGFs produced in the leading domain in response to Wnt signalling activate FGFR signalling in an adjacent trailing domain, where there is less inhibition of FGFR signalling (Matsuda et al., 2013). Previous studies have shown that the balance of Wnt and FGF signalling influences when and where proto-neuromasts form and that this can influence the pattern of neuromast deposition by the migrating PLLp (Matsuda et al., 2013).

Migration of neuromasts along the lateral line is also defined by the expression of SDF1a (stromal-derived factor 1 alpha a, or CXCL12a). SDF1 is a potent chemo-attractant, to which the CXCR4b and CXCR7 receptors respond (Dambly-Chaudiere et al., 2007). The relationship between SDF1a, CXCR4b and CXCR7 expression in teleost lateral line development has been investigated extensively (David et al., 2002, Haas and Gilmour 2006, Dambly-Chaudiere et al., 2007, Xu et al., 2014). Both the SDF1a and CXCR4b molecules are involved in a variety of diverse cell migratory pathways in different model systems including migration of dentate granule cells in mouse (Bagri et al., 2002), neural and sensory axon path-finding (Miyasaka et al., 2007, Sato et al., 2007), and formation of muscle in zebrafish (Chong et al., 2007). With previous accounts of migration of other cell types like mast cells or neutrophils (Beccetti and Arcangeli, 2010, Planttner and Klauke, 2001), being dependent on Ca^{2+} entry via Ca^{2+} channels, it is highly possible that in the lateral line system in zebrafish SDF-1 stimulated migration of neuromasts is sensitive to Ca^{2+} channels. Accordingly a previous report of Schmidt et al. (Schmidt et al., 2011) SDF-1 activates Ca^{2+} channels during its induction of platelet migration. However, a direct link between calcium channels and SDF-1a in zebrafish neuromast migration is yet to be established.

Our study not only characterized the expression pattern of *Cacna2d1* and its role in neuromast development but also opens a new idea about the involvement of two major signalling pathways (calcium signalling and SDF/CXCL12) affecting/influencing each other in the lateral line development in the zebrafish. More thorough research will be necessary to study

the two systems and other possible mechanisms explaining the role of *Cacna2d1* in zebrafish lateral line development.

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7. APPENDIX

7.1. Abbreviations

5-HT	5-hydroxytryptamine
5-HTP	5-hydroxytryptophan
AADC	Aromatic amino acid decarboxylase
AA	Ascorbic acid
ALL	Anterior lateral line
AP	Alkaline phosphatase
BCA	Bicinchoninic Acid
BCIP	5-Bromo 4-Chloro 3-indolyl Phosphate
BH4	Tetrahydrobiopterine
BSA	Bovine serum albumin
CaMKII	Ca ²⁺ -calmodulin-dependent protein kinase II
cDNA	Complementary DNA
CNS	Central nervous system
Cy	Cyclophosphamide
DASPEI	(2-(4-(dimethylamino)styryl)-N-ethylpyridinium iodide)
DEPC	Diethylpyrocarbonate
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DOX	Doxycycline
Dpp	Day post plating
DR	Dorsal raphe
EB	Embryoid bodies

ESC	Embryonic stem cells
FACS	Florescence activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
HyB	hybridization buffer
hpf	Hour post fertilization
HVA	High-voltage activated
iPS	Induced pluripotent stem cells
LIF	leukemia inhibitory factor
Lmx1b	LIM homeobox transcription factor 1 β
LSD	Lysergic acid diethylamide
LTCCs	L-type Ca ²⁺ channels
LVA	Low-voltage-activated
miPS	Mouse induced pluripotent stem cells
MAO	Monoamine oxidase
MEFs	Mouse embryonic fibroblasts
MHO	Midbrain-hindbrain organizer
MMLV	Murine Leukemia Virus Reverse Transcriptase
MnR	Median raphe nucleus
MO	Morpholinos
NBT	Nitro Blue Tetrazolium
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

PDL	Poly-D-Lysine
PFA	Paraformaldehyde
PKC	Protein kinase C
pLL	Posterior lateral line
PTU	1-Phenyl-2-thiourea
PVDF	Polyvinylidenefluorid
RA	Retinoic acid
REFs	Rat embryonic fibroblasts
riPS	Rat induced pluripotent stem cells
RMg	Raphe magnus nucleus
ROb	Raphe obscurus nucleus
RPa	Raphe pallidus nucleus
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulfate
SDF1a	Stromal-derived factor 1 alpha a
SHH	Sonic hedgehog
SC	Superior colliculus
SSCT	Saline-sodium citrate with tween20
SSRIs	Selective serotonin reuptake inhibitors
TAE	Tris/Acetate/EDTA
Taq	Thermus aquaticus
TE	Tris EDTA buffer
TEMED	N,N,N',N'-Tetramethylethylenediamine
TPH	Tryptophan hydroxylase
Tris	Tris(hydroxymethyl)aminomethane

VDCCs	Voltage-dependent calcium channel
VMAT2	Vesicular monoamine transporter 2
Vta	Ventral tegmental area
WB	Western blot
WT	Wild type
YSL	Yolk syncytial layer

8. DECLARATION

Hiermit erkläre ich, dass ich die vorgelegte Dissertation mit dem Titel „Targeted differentiation of ES cells into serotonergic neurons“ selbständig und nur unter Verwendung der angegebenen Hilfsmittel angefertigt habe.

Das Weiteren erkläre ich, dass ich mich nicht anderweitig um einen Doktorgrad beworben habe und auch keinen entsprechenden Doktorgrad besitze. Ferner erkläre ich, dass ich von der dem angestrebten Verfahren zugrunde liegenden Promotionsordnung der Mathematisch/Naturwissenschaftlichen Fakultät I vom 06.07.2009 Kenntnis genommen habe.

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Berlin, den 06.05.2014